IMMUNOLOGICAL STUDIES ON ANTIBODIES AGAINST HEAT SHOCK PROTEINS IN AUTOIMMUNE DISEASES

DISSERTATION

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BY

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To My

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CERTIFICATE

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C O N T E N T S

ABSTRACT

Stress or heat shock proteins are major immune targets in broad spectrum of infectious diseases. Of particular interest to immunologist and rheumatologist is the convergence of data in several fields that suggests that stress proteins in microorganism, commonly infecting humans, may be a trigger of humoral and cellular autoimmune responses and consequent overt autoimmune disease expression. The stress proteins of Mycobacterium tuberculosis and other bacteria are close homologues to that of mammals and may be involved in the pathogenesis of autoimmune diseases. The capacity of stress proteins to bind to multiple proteins and nucleic acids that have been damaged by cell stress or infection may result in the formation of highly immunogenic particles that drive the immune system to produce the marker autoantibodies and linked sets of autoantibodies characteristic of SLE and other systemic autoimmune diseases.

Many attempts have been made to identify the antigens (self/non-self) responsible for the autoantibody production crossreactive with normal constitutents of human cells. The possible involvement of mycobacterial heat shock proteins in the autoantibody production have been indicated but their role in its pathogenesis is not yet clear.

In the present study attempts have been made to probe

the binding of SLE autoantibodies against intracellular protein antigen(s)/heat shock proteins of Mycobacterium tuberculosis ($H_{37}Rv$). The intracellular protein antigens of Mycobacterium tuberculosis were isolated. The immuno-crossreactivity of SLE autoantibodies against mycobacterial cellular protein antigens as well as 70 kD heat shock protein was probed by ELISA and Western blot assay. Furthermore, their specificity was ascertained by competition inhibition ELISA. Inhibition results revealed a greater magnitude of binding for SLE autoantibodies with intracellular mycobacterial heat shock proteins as compared with native DNA. The specificity of anti-DNA antibodies as revealed by inhibition ELISA, was found to be in the order of MTSE > ssDNA > ds DNA > RNA.

70 kD heat shock protein is an immunodominant antigen of Mycobacterium tuberculosis. A large portion of the immune response to infecting mycobacteria appears to be directed at two particular protiens 70 kD, and 65 kD. Hsp 70 was thus isolated by electroeluting the 70 kD band from an electrophoresed 5-20% gradient SDS-polyacrylamide gel. The interaction of hsp 70 kD was probed with Protein A- Sepharose isolated SLE IgG, where a binding of high magnitude was observed. Western blot assay also exhibited a strong recognition of immunoaffinity purified SLE IgG *(anti-DNA antibodies) by the 70 kDa heat shock proteins.*

The retention of cross reactivity of autoantibodies with hsp 70 kD after immunoaffinity purification further augments the idea of an alternate antigen for the induction of autoantibodies cross reactive with native DNA. Thus, the possible involvement of mycobacterial antigens (hsp or non hsp) in SLE autoantibody induction can be inferred from the present studies.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BSA	Bovine serum albumin
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
Hsp	Heat shock protein
IgG	Immunoglobulin G
µg	Microgram
µl	Microlitre
MQ	Milli Q
MTCF	Mycobacterium tuberculosis culture filtrate
MTSE	Mycobacterium tuberculosis sonic extract
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
ssDNA	Single stranded deoxyribonucleic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane

Introduction

When the immune response against microorganisms are analysed, most of the protein antigens involved, of different pathogens, are found to belong to heat shock protein (hsp) families. The ubiquitous distribution of hsp and their high sequence conservation pose interesting challenges to the immune system. On one hand, the omnipresence of epitopes shared by all pathogens may provide the immune system with a universal signal for infection. Furthermore, the expression of hsp by transformed, or otherwise stressed cells could facilitate their rapid and efficient identification through a common mechanism. On the other hand, epitopes shared by the predator and the host may also furnish a link between infection and autoimmunity, which under unfavourable conditions, may trigger deleterious responses (Kaufmann, 1990).

Cultured cells or whole organism are found to respond to a wide spectrum of stresses, which includes heat shock, nutrient deprivation, oxygen radicals, metabolic disruption etc., by synthesizing a small group of proteins namely, the stress proteins. The best studied of these stresses is the heat shock (Lindquist, 1986; Lindquist and Craig, 1988; Welch, 1989) where a sudden increase in temperature induces increased synthesis of shock proteins, hence the name heat shock proteins.

The stress proteins appear to provide the cell with the

protection during or after recovery from the environmental insults. In all organisms the induction of hsp is remarkably rapid and intense in keeping with the notion that it is an emergency response. There is a striking relationship between the induction temperature and the organism's environment. In different organisms the response is induced at different temperatures and each organism is expected to cope with such temperature in its natural environment. As in fruit fly Drosophila melanogaster, induction occurs between 33-37°C which is the common temperature on warm summer days (Lindquist, 1980). In thermophilic bacteria growing at 50°C the proteins are induced when temperatures are raised to 60°C (Daniel et al., 1984) and in mammals they are induced at fever temperature (Li and Laszlo, 1985).

The heat shock response is the most highly conserved genetic system known existing in every organism in which it has been sought from plants to animals. Thus, heat shock proteins are remarkable in their evolutionary conservation (Lindquist and Craig, 1988; Garsia et al., 1989; Hickey et al., 1989; Jindal et al., 1989). Stress protein families are also composed of proteins that are related to stress proteins in sequence but whose levels are not altered by stress.

The exact mechanism by which the cell recognizes a

change in its environmental circumstances and activates the stress response is still unclear. One possible way which is shared by many treatment/ agents inducing a response is their ability to promote protein denaturation and / or aggregation (Ananthan et al., 1986; Edington et al., 1989). Whether this is the only possible mechanism for the initiation of response remains questionable. Different changes take place in cells after stress. After heat shock, an arrest in the cell cycle and a cessation of most activities involved with cellular proliferation (Westra and Dewey, 1971; Zeuthen, 1971) changes in plasma membrane mediated events, which include-

(a) alteration in the activities of Na^+/K^+ ATPase (Yi, 1979; Burdon and Cutmore, 1982; Anderson and Hahn,1985).

(b) decrease in insulin and epidermal growth factor binding activities (Calderwood and Hahn,1983).

(c) rapid decrease in intracellular pH (Weitzel et al.,1985; Drummond et al.,1986). Besides these, *morphological alterations have also been described in* mammalian cells following heat shock treatment (Welch and Suhan, 1985; Yost and Lindquist,1986; Welch and Suhan,1986; Arrigo et al., 1988).

Types of Heat Shock Proteins

A variety of hsp families have been discovered which includes hsp 90, hsp 70, hsp 60 and other small molecular weight hsp families. Each heat shock protein' is named according to its approximate mass in thousands of daltons.

Hsp 90 Family

Members of this family range in size from 83 to 90 kD. Sequence analysis of the cloned genes of hsp 90 family demonstrates that the proteins are highly conserved. In most of the mammalian cells, the hsp 90 are abundant at normal temperature and are further induced by heat (Lai et al., 1984; van Bergen en Henegouwen et al., 1987).In D. melanogaster, there appears to be only one gene in this family, hsp 83. In vertebrates, one gene in this family has been found to be encoding a signal sequence to transport the protein across the endoplasmic reticulum. The other members of the vertebrate family appears to be abundant at normal temperature. Within eukaryotes, members of this family display sequence homology. The E. coli shows 36% identity with D. melanogaster hsp 83. A tumour specific antigen, Meth. A has been identified as hsp 90 (Ullrich et al., 1986) localized atleast partly to the cell surface (Srivastava and Maki, 1991).

No enzymatic functions have been described for these proteins, although they have been shown to interact with

protein kinases and steroid receptors (Kulloma et al., 1986) and may play some role in transport or regulation of these proteins (Langer and Neupert, 1991). The hsp 90 proteins of three parasites, Trypanosoma cruzi, Schistosoma mansoni and Plasmodium falciparum have been reported to be antigenic. No bacterial antigens have been described with homology to members of this family.

Evidence is emerging which suggests that members of the hsp 90 class act as molecular chaperones in the mechanism of signal transduction by steroid receptor. In the absence of steroid hormones, the steroid receptor is bound to hsp 90 and is unable to activate transcription of steroid controlled genes. On the contrary, supplementation of steroid hormone displaces the bound hsp 90 and produces a receptor capable of activating transcription (Ellis, 1991).

Hsp 70 Family

This is the most highly conserved and abundant heat shock protein family (Bienz, 1984; Hunt et al., 1985). Hsp 70 of most if not all eukaryotes, is a member of multigene family, whose genes are under a variety of physiological conditions. The amino acid sequence of the human hsp 70 has 50% homology with that of E. coli and 73% identity with that of Drosophila hsp 70. The sequence identity is greater at amino terminal region of the protein which has been suggested to have a conserved ATPase activity. Some of these

proteins are constitutively expressed while some of these are heat inducible and some are glucose regulated (grp 78). These proteins can be found during heat shock in the nucleus, in cytoplasm after heat shock and in endoplasmic reticulum (Velazquez and Lindquist, 1984). Members of hsp 70 family have acidic isoelectric points and similar tryptic peptide patterns in all species examined (Lindquist, 1986). The mammalian proteins bind ATP very tightly, a property that has recently been exploited to purify them (Welch et al., 1985).

dnak gene in E. coli encodes a protein that is related to hsp 70 of eukaryotes (Bardwell and Craig, 1984). Dnak is 50% identical in amino acid sequence to hsp 70 of eukaryotes. This gene is identified as host gene, necessary for lambda DNA replication. The mycobacterial members of this family are highly homologous to each other and to members of the hsp 70 family (Young et al., 1988; Garsia et al., 1989). An immunoreactive 70 kD antigen has been identified in M. leprae, M. tuberculosis and M. bovis (Britton et al., 1986; Watson, 1989). Four members of the human hsp 70 family have been identified, of which hsp 70 is the major heat shock protein (Watowich and Morimoto,1988).

Hsp 60 Family

Hsp 60 proteins have been extensively characterized in bacteria (E. coli) and recently their eukaryotic homologues

have been identified (Jindal et al.,1989; Mizzen et al., 1989). The amino acid sequence of hsp 60 has been found to be highly conserved between bacteria and human. The sequence identity is found to be in the range of 50 - 60%. Highly immunoreactive proteins of M. tuberculosis and M. bovis BCG are the mycobacterial counterparts to common antigens and that these antigens are homologous to E. coli GroEL heat shock protein (Shinnick et al., 1988; Thole et al., 1988; Young et al., 1988). 65 kD antigen is one of the major immunoreactive proteins of the mycobacteria. This antigen contains epitopes that are unique to a given mycobacterial species as well as epitopes that are common various species of mycobacteria (Gillis and Buchanan, 1982; Engers et al., 1985 and 1986; Gillis et al., 1985).

In addition to the shared epitopes, these proteins also display common structural and immunological features. The features are - (i) they can be isolated as homomultimers of 250 - 900 kD composed of approximately 60 kD sub-units in tubercle bacilli (ii) they contain species - specific, genus - specific as well as cross - reactive epitopes (iii) they can be either located in the cytoplasm or exposed to the cell surface depending on particular species within the genus examined (iv) they are major immunogens and antigens during infection with the corresponding bacteria (Hoiby, 1975; Sompolinsky et al., 1980a and b; deBruyn et al., 1987;

Thole et al., 1987). Atleast four regions of more than ten amino acids in length are found to be identical in man and mycobacteria.

Small Hsp Family

This family belongs to a very diverse group. Different organisms have different numbers of small hsp family. The molecular weight ranges from 14 to 30 kD (Lindquist,1986; Lindquist and Craig, 1988). Though they are divergent in sequence, they are conserved in their stuctural properties. In Drosophila, the members of this family includes hsp 22,23,26 and 28. The 18 kD antigen of M. leprae appears to be a good T-cell immunogen and antigen in individuals with killed M. leprae vaccine (Dockrell et al., 1989).

GroES Family

In many bacteria including E. coli, the heat shock operon expressing GroEL/hsp 60 also contains a 10 kD protein called GroES (Hemmingson et al., 1988), while in eukaryotes, hsp 60 homologues are not a part of operon and that the GroES homologues have not been described (Jindal et al., 1989). In mycobacteria, the GroES homologue is antigenic.

Functions of Heat Shock Proteins

The heat shock proteins are found to perform a variety of functions. Recent studies on heat shock proteins support the notion that these proteins act as molecular chaperones. There is evidence that these proteins can interact with

polypeptide during a variety of assembly processes in such a way as to prevent the formation of non-functional structures. Heat shock proteins not only mediate assembly processes but also promote the disassembly of proteins that have been damaged as a result of stress. Heat shock proteins bind to interactive surfaces that are exposed by stress, followed by ATP mediated release, which triggers conformational change in the denatured protein that favour correct reassembly.

These proteins possess weak ATPase activity. The amino terminal part of hsp 70 harbors the ATP binding site and the ATPase activity. Chappel et al (1987) suggested the term ATPase core for this domain. Hsp 70 is found to be involved in the post translational translocation of proteins across the membranes. The prokaryotic member of hsp 60 family GroEL protein is found to be involved in the head assembly of phage λ (Friedman et al., 1984) and tail assembly of T_5 (Tilly and Georgopoulos, 1982) as well as in T_4 head assembly (Georgopoulos et al., 1972).

Stress proteins have been known to bind a number of different molecules. For example, hsp 90 has been found to interact with steroid hormone receptor, with viral and cellular kinases and with actin and tubulin. Members of the hsp 70 family bind to DNA duplication complexes, clathrin baskets, the cellular tumor antigen p^{53} and immunoglobulin

heavy chain.

In eukaryotic cells, these proteins can be induced in vitro following infection by diverse viruses including by paramyxoviruses (Peluso, 1978) and adenoviruses (Nevins et al., 1982). Some stress proteins including hsp 90 and hsp 70 have been found to accumulate abnormally high in transformed cells (Bensuade, 1983; Macnab et al., 1985).

Immunoreactivity of Heat Shock Proteins

The heat shock proteins are found to be the target of immune response in many bacterial and non-bacterial infection. The possible explanation for this includes -

(a) hsp are abundant cellular proteins

(b) have conserved epitopes that may prime the host for an immune respose to these proteins

(c) may be preferentially processed for presentation due to either structural or functional features.

Regarding their abundance, hsp's are found to accumulate to even higher levels in bacteria undergoing stress. Hsp 70, 60 and GroES account for more than 15% of the total cell protein in heat shocked cells (Neidhart et al., 1984). Mycobacteria grown in zinc deficient media, the hsp 60 accounts for around 9% of the total cell proteins (deBruyn et al., 1987). It is not only the change in temperature that occurs upon infection which induces protein

synthesis, other factors may also stimulate production of heat shock proteins. It is the stress that is inherent inside the lysosome of macrophage that might help to induce the synthesis of these proteins to high levels.

Immunological priming

Due to their conserved nature, it is possible that the infection by one pathogen might prime the host for an immune response against a second pathogen. This suggests that the immune response should be directed primarily against conserved epitopes. But this is not the case, as is found that the humoral immune response to the heat shock proteins is directed predominantly towards non-conserved epitopes (Newport et al., 1988). On the other hand, the T- cell that recognize either conserved epitopes of the mycobacterial hsp 60 or hsp 70 homologues can be readily isolated (Young, 1990). Thus, the heat shock proteins of pathogenic microorganism can elicit humoral and cellular immune respose to epitopes that are shared with their host. Such immunoreactivity could play important roles in pathogenicity and autoimmune consequences of infections.

Structural and functional considerations

Some members of the hsp 70, 90 and 60 families are found to be surface accessible as is seen in certain cells undergoing heat shock or other stresses. They express heat shock proteins on their surfaces or secrete them (deBruyn et

al., 1987; Jarjour et al., 1989; Koga et al., 1989). Perhaps, because of these structural features hsp can be efficiently processed and presented on the macrophage surfaces and hence becomes readily available for interaction with other components of the immune system. Presentation might also be facilitated by the ability of hsp 70 and hsp 60 to bind to denatured proteins and perhaps a pathogen's heat shock protein could be 'coprocessed' along with other proteins and thereby increase the likelihood of a heat shock protein being presented by the macrophage. Thus the structural and functional features of the heat shock proteins make them particularly favourable candidates for processing and presentation to the immune system and hence potential immunogen.

Autoimmune Diseases and Hsp

The immune system has tremendous discrimination potential of self and non-self. However, this enormous recognition potential includes possible interaction with self components, some of which appear to be essential for the regulation of the immune functions, (Jerne, 1974), whereas other can be pathogenic particularly if expressed at high level, leading to autoimmune diseases.

Many theories have been proposed to explain the origin of autoimmune responses. For example, it has been postulated that immune responses directed against host

antigen could result from genetic predisposition, random B-cell activity from host and foreign antigen or modification of host protein as a consequence of infection and drug administration. It is now apparent that normal immune system indeed have the capacity to produce antibodies to a variety of self antigens. This new orientation of thinking about autoimmunity raises many tantalizing questions whose answers can only be speculated.

Among the various autoimmune diseases, systemic lupus erythematosus (SLE) is a prototype autoimmune disease of unknown etiology and characterized by the presence of heterogenous population of circulating antibodies of intracellular proteins and nucleic acids.

Stress proteins (hsp), are major immune targets in broad spectrum of infectious diseases and have extremely close homologues in man. Infection entails 'stress' for both the microorganism as well as the host, therefore, it would appear to be an ideal stimulus for the emergence of autoreactive T-cells and B-cell reponses against host stress proteins.

Different autoimmune diseases are associated with humoral responses to self hsp. There are also evidences linking mycobacterial infection with humoral autoimmunity (Shoenfeld and Isenberg, 1988). Hyper-

gammaglobulinemia is found to be associated with active tuberculosis, and tubercle bacilli are extremely potent adjuvants for induction of polyclonal B-cell activation. Patients with pulmonary tuberculosis are found to develop rheumatoid factor and many of the same antinuclear antibodies characteristics of patient with SLE. The behaviour of hsp 70 and ubiquitin in the cell raises the possibility that stress proteins may be key ingredient in the 'immunogenic particle' concept (Tan et al., 1988) of the origin of antinuclear and other antibodies , as has been suggested previously (Muller et al., 1988; Inque, 1989).

Thus, hsp 70 associates itself with proteins in the nucleus and nucleolus in a cell cycle dependent fashion (Milarski et al., 1989) and migrates to different cell compartment and bind to a variety of nuclear cytoplasmic protein in various infected cells (Nevins, 1982; Welch et al., 1985; La Thangue and Lachmann, 1988; White et al., 1988; Pallas et al., 1989; Sawai and Butel, 1989; Welch and Suhan, 1989). Also because stress proteins of bacteria are homologous with that of host, thus there exist the possibility in qenetically susceptible individual for a constant risk of autoimmune disease development due to the failure of mechanism for self/nonself discrimination through molecular mimicry

(Oldstone, 1987). That these proteins actually play a role in autoimmune processes is suggestive for the fact that autoreactivity in chronic inflamatory arthritis involves at least initially, cells recognizing epitopes of stress protein hsp 60.

The immunodominant proteins of infecting microorganism are homologues of hsp in eukaryotes (Hedstrom et al., 1987; Young et al., 1988). The evidence for the existence in SLE of a previously undescribed autoantibody to the 73 kD constitutively expressed members of hsp 70 family is consistent with the concept of molecular mimicry as a mechanism of autoantibody induction in this disorder.

Antibodies to members of hsp 90 family have been described in serum from patients suffering from systemic lupus erythematosus (SLE) (Minota et al., 1988a). Stress antigens such as hsp 70 are also found to react with antibodies from these patients. Also autoantibodies to hsp 70 were detected in a minority of sera from patients with other rheumatic or viral diseases, but not in normal sera. (Minota et al., 1988b). However, recent studies have shown a similar frequency of anti hsp 70 antibodies in patients with SLE and normal human subjects (Kindas-Mugge, 1993). Possibly this may be generated in the course of infections, since hsps are among the most dominant immunogens of infectious microorganism (Shinnick,

1991). The increased expression of endogenous hsp occurring during infection and inflammatory processes has been observed in peripheral blood cells from SLE patients (Deguchi, 1987; Norton, 1989; Deguchi, 1990). Thus, the anti-hsp immune response could be elicited both by a foreign organism as well as by self components. Similar frequency of anti hsp 70 autoantibodies in SLE and normal subjects can not rule out the possibility that these autoantibodies may contribute to the complex pathogenesis of SLE at least in sub-groups of SLE patients. The elevated levels of IgA anti-hsp 70 antibodies reported by Tsoulfa et al. (1989), and the high titre of IgM antibodies measured in few of SLE patients (Kindas-Mugge, 1993) might indicate such relationship. Thus it can be imagined that the major disturbances of the immune network which occur in autoimmune diseases might also affect the anti-hsp response which then could change from a mechanism of defence to mechanism of self destruction.

Anti MT-hsp 65 antibodies have also been detected in sera of patients with systemic sclerosis (SSc) (Danieli et al., 1992) although its mechanism of induction still remains unanswered. The reaction of MT-hsp 65 can be viewed as the consequence of immunity against epitopes of human hsp 65 which are shared by mycobacterial counterpart. The two molecules have 45% homology (Jindal et al., 1989). The hsp

autoimmunity thus can be amplified during conditions leading to the synthesis of hsp in sufficient amount for T - cell activiation in the context of HLA-class II molecule. Ischemia, well documented in SLE, might represent the stress responsible for increased hsp expression (Polla, 1988). The correlation between anti hsp antibodies and disease activity in SSc as well as normal values in the group of SLE (Danieli et al., 1992) are remarkable findings which indicate that the immune reaction is not a part of acute phase response but may be reflection of pathogenic mechanism.

A recent study by Koga et al (1989) provides evidence that stressed host cell can serve as targets for T-cells against hsp 65. Although it is often assumed that only $CD4^+$ T-cells are activated by intracellular bacteria, recent evidence suggests a role for $CD8^+$ T-cells in these infections (Kaufmann, 1988). This idea was supported by work of Rees et al. (1988), who isolated a $CD8^+$ T-cell line from the pleural effusions of tuberculosis patients that cross reacted with hsp 70 conjugates from M. tuberculosis, E.coli and humans.

The general concept that can be formulated regarding the role of hsp in autoimmunity from the different findings are summarized as follows-

(a) At least some T cells and antibodies against epitopes shared by bacterial and human hsp's have evaded tolerance mechanism and hence are present in many individual.

(b) These T-cells and/or antibodies are activated during microbial infection by contact with microbial hsp's.

(c) Self epitopes of hsp's are expressed by host cells either alone or in the context of MHC molecules that suffer under certain stress conditions.

(d) Recognition of these stressed host cells by T-cells and/or antibodies with specificity of shared epitope, trigger an autoimmune response.

Objective of Present Study

In the present study, attempts were made to probe the possible involvement of intracellular mycobacterial and heat shock proteins in the induction of human autoantibodies. The pathogenic strain of Mycobacterium tuberculosis ($H_{37}Rv$) was cultured on Sauton's medium. The intracellular proteins from the bacilli were obtained by sonication. Gradient SDS-PAGE was employed in order to monitor the wide spectrum of intracellular proteins. The 70 kD heat shock protein of the Mycobacterium was detected by gradient SDS-PAGE and isolated by electroelution. The immunological investigations were carried out by employing ELISA and Western blotting. The immunologic crossreactivity of

naturally occurring SLE anti-DNA autoantibodies with total mycobacterial sonicate (intracellular proteins) as well as 70 kD heat shock protein was probed by ELISA. The binding of immuno-affinity purified anti-DNA antibodies with 70 kD heat shock protein was ascertained by employing Western blotting.

Experimental

A. MATERIALS

1. Chemicals

Bovine serum albumin, calf thymus DNA, agarose, Coomassie brilliant blue R-250, ethidium bromide and anti-human IgG alkaline phosphatase conjugate were purchased from Sigma Chemical Company, U.S.A. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm in diameter) were obtained from NUNC, Denmark. Tween 20, ammonium persulphate, acrylamide, bis-acrylamide, N,N,N,'N'- tetramethylethylene diamine were from Bio-Rad Laboratories U.S.A., Sephadex G-25, Sephadex G-200, Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. A colorigenic substrate para- nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, Delhi. Citric acid, magnesium sulphate, asparagine, dipotassium hydrogen phosphate, ferric ammonium citrate and glycerol were obtained from Qualigens, India. Sodium pyruvate was obtained from SRL, India. All other chemicals were of highest grade available.

2. Equipments

ELISA microplate reader MR-600 (Dynatech, U.S.A.), ELICO pH meter model L1-10T, Shimadzu spectrophotometer UV-240, Slab gel electrophoresis (Bio-Rad Laboratories, U.S.A.) and Gradient mixer (Pharmacia Fine Chemicals, Sweden) and high speed refrigerated centrifuge (Avanti 30,

Beckmann, U.S.A.) and sonicator (Braun-Sonic, Melsunger, Germany) were the major equipments used in this study.

B. METHODS

1. Purification of DNA

Calf thymus DNA was purified free of proteins and single stranded regions by the method of Ali et al., (1985). DNA solution (2 mg/ml) in 0.1xSSC buffer (0.015 M sodium citrate, pH 7.3 containing 0.15 M sodium chloride) was mixed with equal volume of chloroform-isoamyl alcohol (24:1 ratio) in a stoppered glass cylinder. The cylinder was sealed and the contents were mixed end-to-end gently for one hour. The DNA in aqueous phase was separated from the organic layer and the extraction process was repeated. DNA was precipitated with two volumes of cold ethanol and were subsequently collected on a glass rod. The precipitate was air dried to remove traces of ethanol. The DNA was dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM $ZnCl_2$. The sample was then incubated with single strand specific enzyme, nuclease S_1 (250 units/mg DNA) at 37°C for 30 minutes. The reaction was terminated by adding one tenth volume of 0.2 M EDTA, pH 8.0. The purified DNA was extracted thrice with chloroform -isoamyl alcohol and reprecipitated with cold ethanol. Finally the DNA was dried and dissolved in required buffer.

2. Determination of DNA concentration

DNA was estimated by two methods

(a) Colorimetric assay

(b) Fluorimetric assay

(a) Colorimetric estimation of DNA

Colorimetric estimation of DNA was carried out according to Burton (1956) using diphenylamine reagent.

(i) Diphenylamine reagent

50 ml of glacial acetic acid was added to 750 mg of recrystallized diphenylamine. To this, 0.75 ml of concentrated sulphuric acid was added. The reagent was prepared fresh before use.

(ii) Procedure

To 1.0 ml of DNA sample (with varying concentration of DNA), 1.0 ml of 1 N perchloric acid was added and the tubes were incubated for 15 minutes in a water bath maintained at 70°C. To this 100 µl of 5.43 mM acetaldehyde was added followed by 2.0 ml diphenylamine reagent. The tubes were vortexed and then allowed to stand at room temperature for 16-20 hrs for color development. Absorbance was monitored at 600 nm. The concentration of DNA in unknown sample was determined from the standard plot constructed with purified calf thymus DNA (Figure 1).

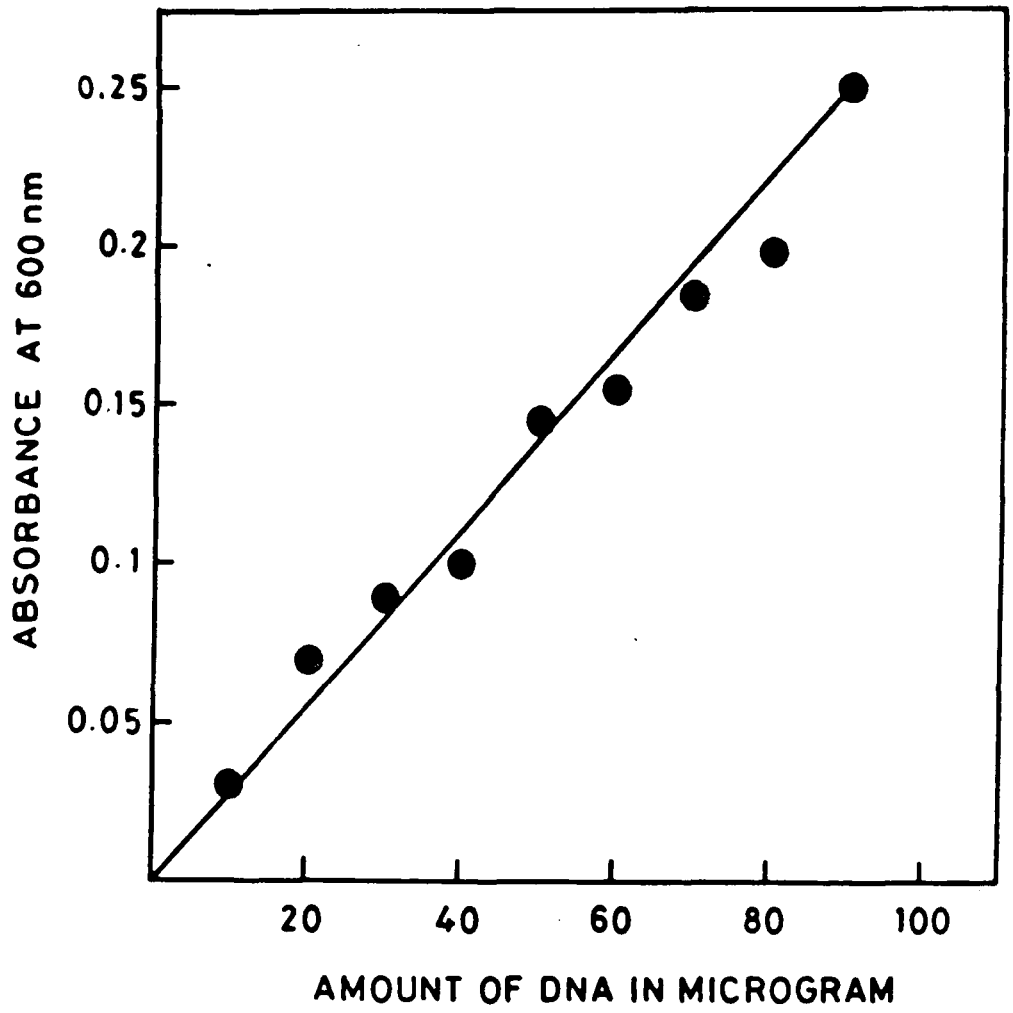

Figure 1. Standard plot for the colorimetric estimation of DNA.

(b) Fluorimetric assay of DNA

Fluorimetric estimation of DNA was carried out according to Lebarca and Paigen (1979) using Hoechst dye. The Hoechst 33258 dye [C2-{2-(4 hydroxy-phenol)-6-benzimidazolyl-6(1-methyl-4 piperozyl)}) interacts with DNA with many fold increase in fluorescence.

a) **Reagents**

(i) 0.05 M sodium phosphate (pH 7.4) containing 0.002 M EDTA and 2 M sodium chloride.

(ii) 200 µg/ml of Hoechst 33258 in deionized water. Working dye solution was prepared by diluting (ii) 200 fold in solution (i), when needed.

b) **Method**

0.1 - 1.0 µg of DNA in 0.1 ml final volume was mixed with 2.4 ml of working dye solution. The mixture was then incubated for 5 minutes at room temperature and the fluorescence was read at 460 nm.

3. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al. (1951) as described below.

(a) Folin-Ciocalteu reagent

The reagent was purchased from C.S.I.R. Centre for Biochemicals, Delhi. It was diluted to 1:4 with distilled water before use.

(b) Alkaline copper reagent

The composition of alkaline copper reagent were

(i) 2.0 percent sodium carbonate in 0.1M sodium hydroxide.

(ii) 0.5 percent copper sulphate in 1.0 percent sodium potassium tartrate.

The working reagent was prepared fresh by mixing components (i) and (ii) in the ratio 50:1.

(c) Procedure

To 1.0 ml of protein samples were added 5 ml of alkaline copper reagent. The tubes were vortexed and allowed to stand at room temperature for 10 minutes. One ml of Folin-Ciocalteu reagent was added and the reaction was allowed to proceed for 30 minutes. Absorbance was monitored at 660 nm. Standard plot was constructed by using bovine serum albumin. From the standard plot the concentration of protein in unknown sample was determined (Figure 2).

4. Isolation of IgG

Protein A - Sepharose is a popular and simple tool in the purification of IgG and its subclasses. Commercially obtained Protein A - Sepharose CL-4B (1.5 gm) was swelled in 0.01 M phosphate buffered saline (PBS), pH 7.4 at room temperature for 48-72 hr. The swelled gel (about 5.0 ml) was allowed to pack in a small column measuring 0.9 cm x 15 cm by gravity sedimentation. The column was washed once

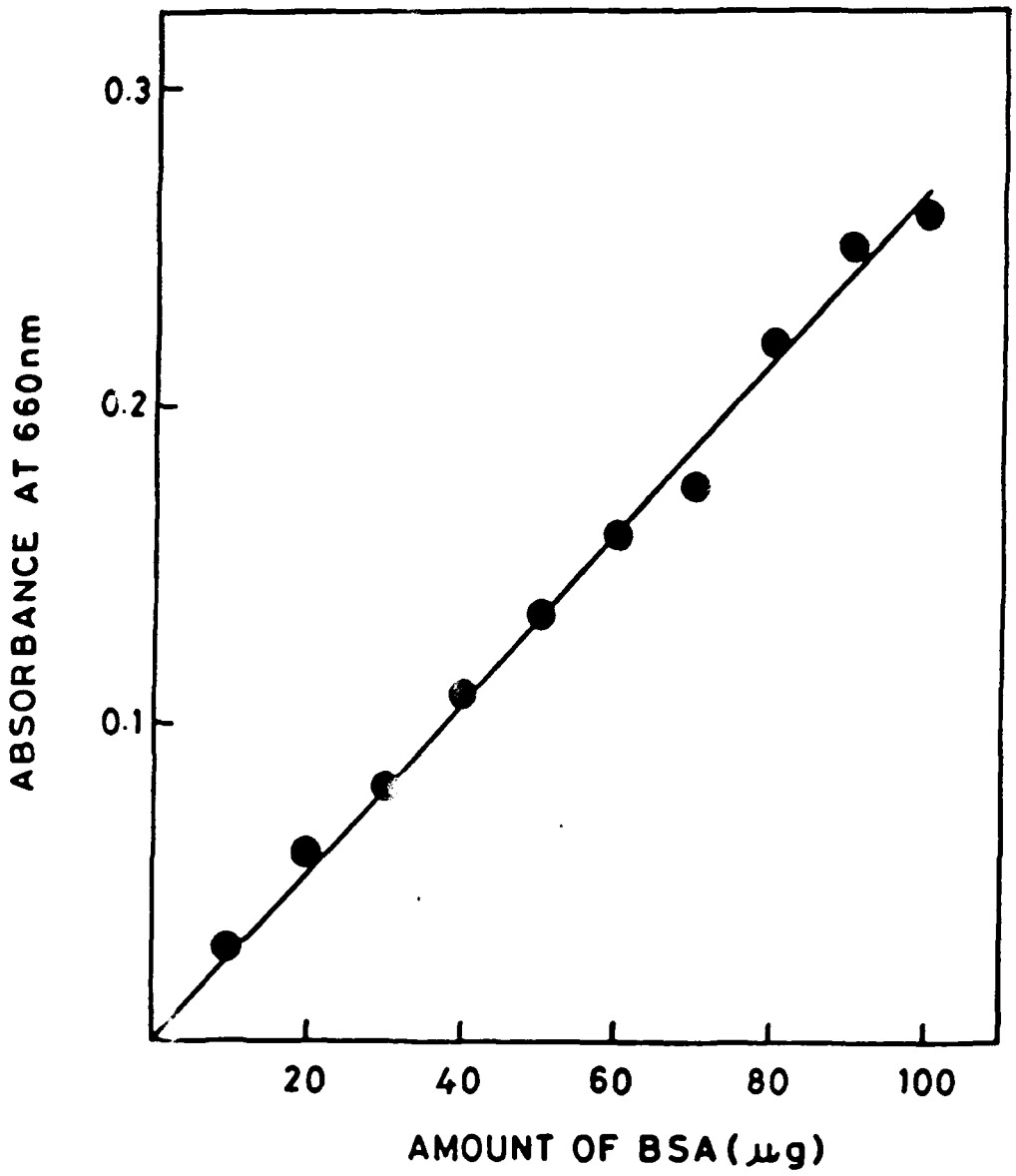

Figure 2. Standard plot for the colorimetric estimation of protein.

with 0.1 M sodium citrate, pH 3.0, to elute any previously bound material and then equilibrated with five volumes of PBS. Serum was diluted in an equal volume of PBS and passed through the column at a rate of 20 ml/hr. The unbound protein was eliminated by washing and the bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976). To prevent the effect of acidic pH of elution buffer on IgG , the eluate was collected in 1 ml of 1 M Tris-HCl, pH 8.5. The sample tubes were monitored at 280 nm and the concentration was determined by the relation, 1.4 O.D. $_{280}$ = 1.0 mg IgG/ml.

5. Immunoaffinity Purification of Immunoglubulin G

(a) CNBr activation of Sepharose 4B

Fifteen millilitre of supplied Sepharose 4B was suspended in distilled water, filtered and washed with 300 ml of cold distilled water on a sintered glass funnel (porosity G-2). Moist gel (approximately 10 g) in 10 ml of 2M sodium carbonate was kept in ice-NaCl bath kept on magnetic stirrer. To the gel was added one gram of cyanogen bromide in 0.8 ml acetonitrile with gentle stirring. The reaction product was filtered after 12 minutes on sintered glass funnel washed with 400 ml of cold 0.1 M sodium carbonate and resuspended in 10 ml of the same buffer. The unreacted CNBr (drained effluent) was reacted with

ferrous sulphate to convert it into harmless ferrocyanide. All steps were carried out in a fume-hood chamber (Ali et al., 1984).

(b) Poly-L-lysine coupling to activated Sepharose 4B

The method described by Wilcheck (1973) was followed. Immediately after CNBr activation of the gel an equal volume of poly-L-lysine (100 mg/10 ml of 0.1 M sodium carbonate) was added and kept at 4°C for 12 hr with slow stirring. The buffer was drained out, followed by washing with 100 ml each of cold (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral. Finally, the gel was suspended in 40 ml of 0.15 M acetate buffer, pH 4.5. The extent of poly-L-lysine depletion due to covalent coupling with activated Sepharose 4B was quantified as described by Habeeb (1966).

(c) Immunoaffinity purification of anti-DNA antibodies

DNA-[polylysyl - Sepharose 4B] affinity column was prepared according to the method described earlier (Nicotra et al., 1982) with little modification. Twenty millilitre of polylysyl -Sepharose 4B was packed in a glass minicolumn (1.6 cm x 50 cm) and equilibrated with acetate buffer. Purified DNA (12.5 ml of 100 µg/ml in acetate buffer) was loaded onto the gel and unbound material was washed with 50 ml of PBS, pH 7.4. Heat decomplemented SLE serum dialysed against PBS, diluted

to 1:10 and was passed through the column. The unbound antibodies were eliminated by washing and the bound proteins were eluted with a linear ionic strength gradient of 0.15 - 3.0 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. Eluate was collected and monitored at 251 nm, 260 nm, 278 nm and 280 nm. The molar concentration of sodium chloride in fractions was evaluated by conductance measurements.

d) Regeneration of affinity column

The same column was regenerated several times by washing successively with 50 ml each of (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral.

6. Agarose Gel Electrophoresis of DNA

(a) 1.0 percent agarose

Agarose NA (1%) in 30 ml of TAE buffer, pH 8.0 was brought to molten state by boiling. The molten solution was allowed to cool to 50-60°C and was subsequently poured onto a horizontal gel tray of GNA -100 electrophoresis apparatus (Pharmacia, Sweden) and was left at room temperature for complete solidification.

(b) Electrode buffer (TAE buffer)

40 mM Tris-acetate and 2mM EDTA , pH 8.0 .

(c) Sample preparation

The samples of less than 1 µg/ml in low ionic strength

were loaded into the wells. The sample contained one tenth volume of 30% Ficoll, 0.025% bromophenol blue, 0.5 M EDTA and 10 electrophoresis buffer.

(d) Running conditions

30 mA for 2 hours.

(e) Staining

The electrophoresed gel was stained with ethidium bromide (1 µg/ml) and the bands were visualised on a UV- transilluminator.

7. Polyacrylamide Slab Gel Electrophoresis for Proteins

Polyacrylamide slab gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared.

(i) Acrylamide-bisacrylamide (30:0.8)

Thirty gram of acrylamide and 0.8 g bisacrylamide were dissolved in a total volume of 100 ml. The solution was mixed thoroughly, filtered and stored at 4°C in an amber colored bottle to prevent photopolymerization.

(ii) Resolving gel buffer - 3 M Tris-HCl, pH 8.8

Stock solution was prepared by dissolving 36.3 gm Tris base in 48.0 ml of 1N HCl. The contents were mixed properly and the pH was adjusted to 8.8. The final volume was brought to 100 ml.

(iii) Stacking gel buffer - 0.5 M Tris-HCl, pH 6.8

To 40 ml of distilled water 6.05 gm of Tris base was dissolved. The pH was adjusted to 6.8 with 1 N HCl. The final volume was made up to 100 ml.

(iv) Electrode buffer

Tris - glycine buffer (0.025 M and 0.192 M respectively), pH 8.3 containing 0.1 percent SDS.

(v) Sample buffer

6.0 gm Tris was dissolved in 80 ml of distilled water and the pH was adjusted to 6.8 with 0-phosphoric acid. Final volume was made up to 100 ml with distilled water.

To 12.5 of above solution, 19 mg of bromophenol blue and 12 ml of glycerol was added. The contents were mixed thoroughly prior to use.

(vi) Sample preparation

Protein samples were mixed with sample buffer in the ratio of 4:1 (w/w). SDS was added to a final concentration of 2% and protein sample was boiled for 5 minutes prior to loading.

(vii) Procedure

Thoroughly washed glass plates (19 cm x 16 cm), separated by 1.0 mm thick spacer was sealed with 1% agarose. A 5-20% gradient gel was layered with 2.5% stacking gel according to recipe given below.

Recipe for 5-20% Gradient Gel (Total volume 24 ml)

Solution	**5%**	**20%**
Acrylamide-bisacrylamide	2.0 ml	8.0 ml
Resolving gel buffer	1.5 ml	1.5 ml
10% SDS	120 µl	120 µl
1.5% ammonium persulphate	600 µl	600 µl
Distilled water	7.774 ml	1.774 ml
TEMED	6 µl	6 µl

Recipe for 2.5% Stacking gel (Total volume 10 ml)

Acrylamide - bisacrylamide	1.25 ml
Stacking gel buffer	2.5 ml
10% SDS	100 µl
1.5% ammonium persulphate	500 µl
Distilled water	5.65 ml
TEMED	7.5 µl

The reagents were mixed in the same order as is shown above and were poured into the gradient former. A 5-20% of the gradient gel was formed between the glass plates. The gel was then allowed to polymerize at room temperature. The protein samples were loaded into the wells and the electrophoresis was carried out for 6-8 hr at 70 Volts. The gel was stained overnight with Coomassie brilliant blue R

250. Destaining was carried out in a mixture of 10% acetic acid and 10% methanol.

8. Enzyme Linked Immunosorbent Assay (ELISA)

The presence of naturally occurring autoantibodies in the sera of patients with SLE against various nucleic acid and mycobacterial antigens were detected and quantitated by ELISA using polystyrene flat bottom microtiter plates as solid phase. The method described by Alam and Ali (1992) and Arif et al. (1994) was followed for the assay.

(a) Buffers and Substrate

(i) Tris buffered saline (TBS)	0.01 M Tris 0.15 M NaCl pH 7.4
(ii) Tris buffered saline-Tween-20 (TBS-T)	0.02 M Tris 0.144 M NaCl 0.00268 M KCl Tween-20 -500 µl/lit. pH 7.4
(iii Coating buffer	0.015 M sodium carbonate 0.035 M sodium bicarbonate pH 9.6
(iv) Substrate buffer	0.015 M sodium carbonate

		0.035 sodium bicarbonate pH 9.6 containing 0.002 M magnesium chloride.
(v)	Substrate	500 µg p- nitrophenyl phosphate per ml of substrate buffer.

(b) Procedure

Polystyrene plate was coated with 100 µl of mycobacterial protein antigens (50 µg/ml) in carbonate-bicarbonate buffer, pH 9.6 for two hours at room temperature followed by overnight incubation at 4°C. This was followed by washing the antigen immobilized plates thrice with TBS-T. The unoccupied sites were blocked by 150 µl of 1.5% BSA in TBS for 5-6 hr. at room temperature. The plates were then coated with varying concentrations/ dilutions of antibody (100 µl) to be tested. The interaction was allowed to occur for 2 hr at room temperature followed by overnight incubation at 4°C. After extensive washing with TBS-T (4 times), the plates were coated with anti-human IgG alkaline phosphatase conjugate and the reaction was allowed to proceed for 2 hr at room temperature. Finally, after washing the plates with TBS-T (4 times), a colorigenic substrate namely p-nitrophenyl phosphate was added to each well (100 µl). After proper color development at 37°C, absorbance of each well was monitored at 410 nm on a Dynatech ELISA

Microplate Reader. Each sample was coated in duplicate and the results were expressed as a mean of A_{test}- $A_{control}$.

For nucleic acid antigens, polystyrene plates were precoated with 100 µl of poly D-lysine solution (50 µg/ml in distilled water) in order to increase antigen immobilization. The plates were washed thrice with TBS to eliminate the unadsorbed bridging molecule and were coated with nucleic acid antigens (100 µl of 2.5 µg/ml stock in TBS). The reaction was allowed to proceed for 2 hr at room temperature, followed by overnight incubation at 4°C. The antigen coated plates were washed thrice with TBS-T and coated with 100 µl/well of poly-L-glutamate (50 µg/ml in TBS) for 2 hr at room temperature. The plates were subjected to washing and the rest of the steps followed were exactly the same as described above.

9. Competition ELISA

The specificity of naturally occurring SLE autoantibodies against nucleic acid and mycobacterial antigens was investigated by competition inhibition experiments. For the assay, varying concentrations of competitors were interacted with antibody (serum or purified IgG) at 37°C for 2 hr followed by overnight incubation at 4°C. The reaction resulted in the formation of immune

complex (IC) which was ultimately used in ELISA instead of serum. The inhibition of antigen binding in presence of various inhibitors (competitors) was determined by measuring their ability to reduce the resulting absorbance relative to control assay lacking competitors. The results were expressed as percent inhibition.

10. Immunodiffusion

Ouchterlony double diffusion method was employed for carrying out precipitin reactions, using glass petri dishes, (Tan et al., 1966). Agarose (0.4 percent) solution was prepared in PBS containing 0.1 percent sodium azide. Six millitre of this molten mixture was poured onto a 5 cm x 1.5 cm glass petri dishe. The dishes were allowed to harden at room temperature and stored at 4°C. All sera were decomplemented by heating at 56°C for 30 minutes. Fifty microlitre each of serum and antigen was placed in the wells and the reaction was allowed to proceed for 72 hr in a moist chamber at room temperature. Petri dishes were flooded with 5 percent sodium citrate to remove non-specific precipitin lines. The antigen-antibody precipitin lines were analysed visually and results recorded.

11. Counter Immunoelectrophoresis

Three millilitres of the molten agarose in 25 mM barbital buffer, pH 8.4 was pipetted onto alcohol cleaned

microscopic slides measuring 7.5 cm x 2.5 cm. The gel was allowed to solidify at room temperature. Wells measuring 4 mm in diameter were made at a distance of 5 mm between the two opposite wells. Antigens were placed in the cathodal well, whereas the decomplemented serum in the anodal wells (25 µl in each wells). Electrophoresis was performed for 45-60 minutes in 50 mM barbital buffer, pH 8.4 with a current of 3 mA per slide. The slides were washed with 5 percent sodium citrate to remove the non-specific precipitin lines.

12. Isolation of Mycobacterial Antigen

(a) Organism

Mycobacterium tuberculosis $H_{37}Rv$ strain was obtained from All India Institute of Medical Sciences, New Delhi by passage on Lowenstein-Jensen medium.

(b) Medium

Modified Sauton's medium was used to culture the pathogenic strain. The composition of the medium was as follows.

Asparagine	4.0 gm
Citric acid	2.0 gm
Magnesium sulphate	0.5 gm
Dipotassium hydrogen phosphate	0.5 gm
Ferric ammonium citrate	0.05 gm

Glycerol	35 ml
Sodium pyruvate	05 mg

The above components were dissolved serially and the final volume was made upto one litre with distilled water. The pH was adjusted to 7.4 by addition of dilute ammonium hydroxide solution. The medium was distributed in 4-5 one litre conical flasks (200 ml in each flask) and were subsequently autoclaved at 120°C for 30 minutes.

(c) Procedure

The culture media was inoculated with $H_{37}Rv$ strain and was allowed to grow at 37°C for 10-12 days. The culture was then heat killed at 100°C for 75 minutes and were harvested by centrifugation at 10,000 rpm for 30 minutes at 4°C. The supernatant (MTCF) was then separated from the pellet (bacilli).

13. Sterlization of Antigens in Mycobacterium tuberculosis Culture Filtrate

The supernatant was sterlized by filtration through a 0.45u pore size membrane (Millipore Corp. U.S.A.). The Mycobacterium tuberculosis culture filtrate (MTCF) was fifty fold concentrated and passed through gel filtration PD-10 column (containing G-25) for desalting. The MTCF was then stored at -70°C in smal aliquots.

14. Liberation of Intracellular Proteins from Heat Killed $H_{37}Rv$ Bacilli by Sonication

Heat killed bacilli (pellet) were suspended in sonicating buffer containing 1 mM DTT (dithiothreitol), 1 mM PMSF and 50 µl of beta-mercaptoethanol in Tris buffered saline, pH 7.4). The cell walls of bacilli were disrupted in ice-cold bath by sonication for 15 minutes. The process was repeated thrice at an interval of 5 minutes. The sonicated bacilli was microcentrifuged at 20,000 x g for 5-10 minutes. The protein content of the supernatant (sonic extract) was determined colorimetrically as described earlier using bovine serum albumin as standard. The sonic extract containing a wide spectrum of mycobacterial protein antigens was stored in aliquots at -20°C for further study.

15. Electroelution

The 70 kD heat shock protein antigen appearing in the gradient SDS-PAGE was carefully sliced off and were separately isolated from the gel by electrophoretically transferring the protein bands from the gel in an electroelution chamber equipped with BT-1 and BT-2 membranes (Bio-Rad). The elution was carried out at 150 volts for 5 hr at room temperature. The transfer buffer used was TBS containing 20% methanol (150 mM Tris-HCl,175 mM NaCl, pH 7.4). The electroeluted proteins were dialyzed

against MQ water, lyophilized and stored at -20°C.

16. Western Blotting

The electrophoresed proteins on the gradient gel was transferred onto nitrocellulose membranes (Bio-Rad) according to the method of Towbin et al. (1979). Transfer of proteins from the gel to the NC membranes was accomplished on a Bio-Rad Protean II transfer cell at 80 volts. The ingredients of transfer buffer were 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol and 0.002% SDS, pH 8.3 . The complete transfer of proteins to the membranes was checked by staining a strip by Amido Black. Thereafter, the protein loaded membrane was treated with 1% BSA in TBS-T (w/v) in order to saturate the binding sites on NC membrane. Saturation was accomplished at 4°C overnight. The saturated membranes were treated with affinity purified anti-DNA antibodies and the reaction was allowed to proceed for 2 hr at room temperature followed by overnight incubation at 4°C. Thereafter, the NC membranes were washed with TBS-T for four times where the duration between each wash was 10 minutes. The bound antibodies were then detected by treating the NC membranes with anti-human IgG conjugated to horse-radish peroxidase (1:1500 dilution in TBS) for 1 hr at room temperature. Unbound conjugate was removed by washing the NC membranes

with TBS-T for five times, followed by a final wash with distilled water. The colour was developed by treating the NC membranes with the substrate (12 mg 4-chloro-1-naphthol dissolved in 4 ml methanol, 20 ml TBS, 40 µl H_2O_2) in dark. After colour development, the membranes were washed with water, dried and photographed.

Results

The presence of autoantibodies to self antigens including nucleic acids have been detected in sera of pateints with systemic lupus erythematosus (SLE) (Tan, 1989; Alam and Ali, 1992).

In the present study attempts have been made to probe the binding of SLE autoantibodies against intracellular protein antigen(s) of Mycobacterium tuberculosis ($H_{37}Rv$). The autoantibodies were characterized by direct binding ELISA, competition ELISA, band shift assay and Western blot.

Sera of various patients with SLE as well as of normal humans were tested for the presence of anti-DNA antibodies by CIE and ID. Both the techniques failed to detect precipitating anti-DNA antibodies. Among the various SLE sera tested by direct binding ELISA, only those exhibiting a titer greater than 1:6400 were opted for further studies (Figs. 3 and 4).

The selected SLE sera exhibiting high titer anti-DNA antibodies were further subjected to antigenic specificity study by employing competition-inhibition ELISA. With native DNA as an inhibitor, the competition results showed a maximum of 70 percent inhibition (Fig. 5) in the antibody binding at a maximum inhibitor concentration of 10 µg/ml. Fifty percent elimination in the autoantibody binding was recorded at an inhibitor concentration of 2 µg/ml. As

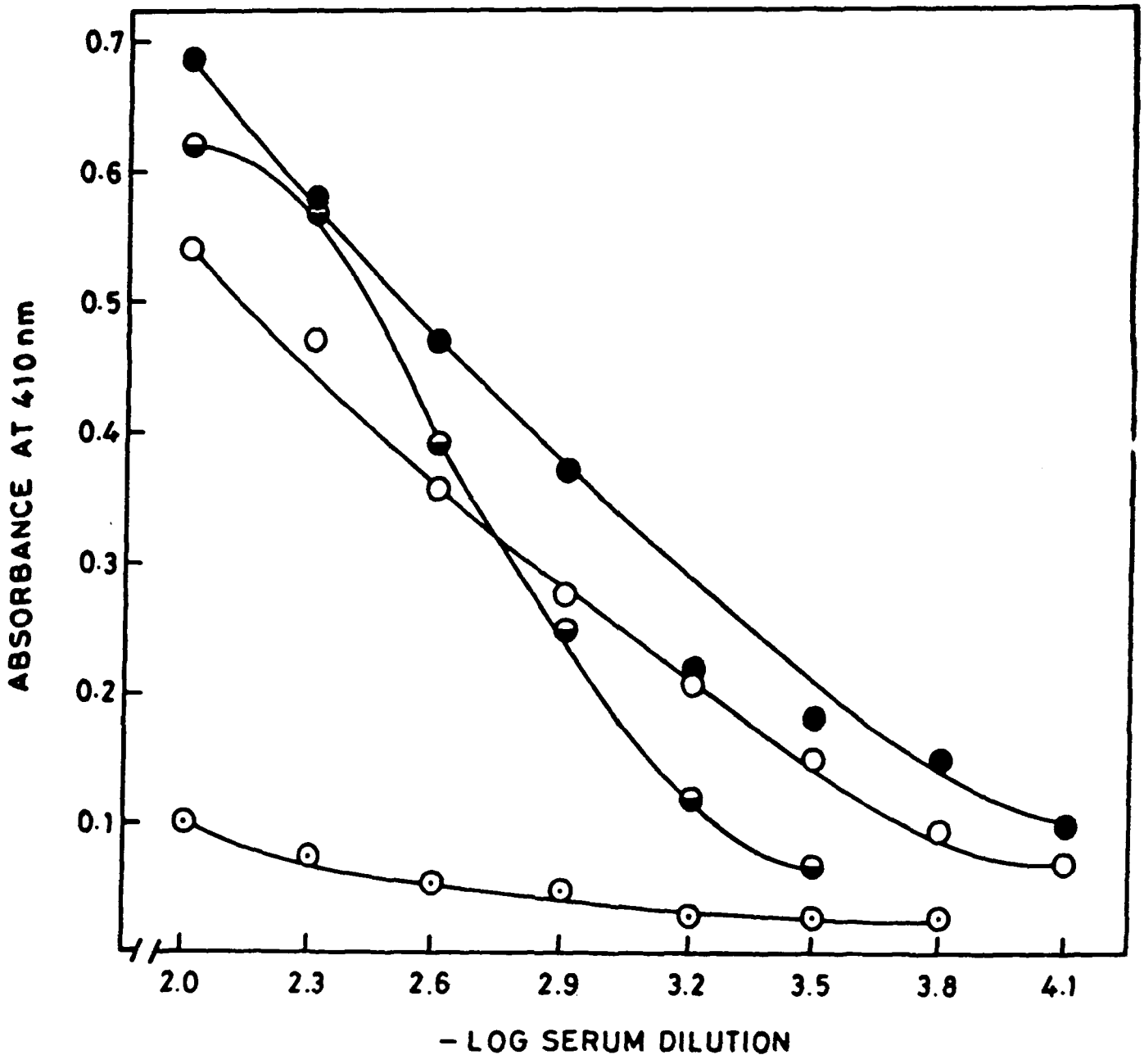

Figure 3. Direct binding ELISA of SLE serum. The microtiter plates were coated with native DNA (2.5 µg/ml).

(●–) SLE serum 1 , (◐–) SLE serum 2
(○–) SLE serum 3 , (⊙–) Normal human sera

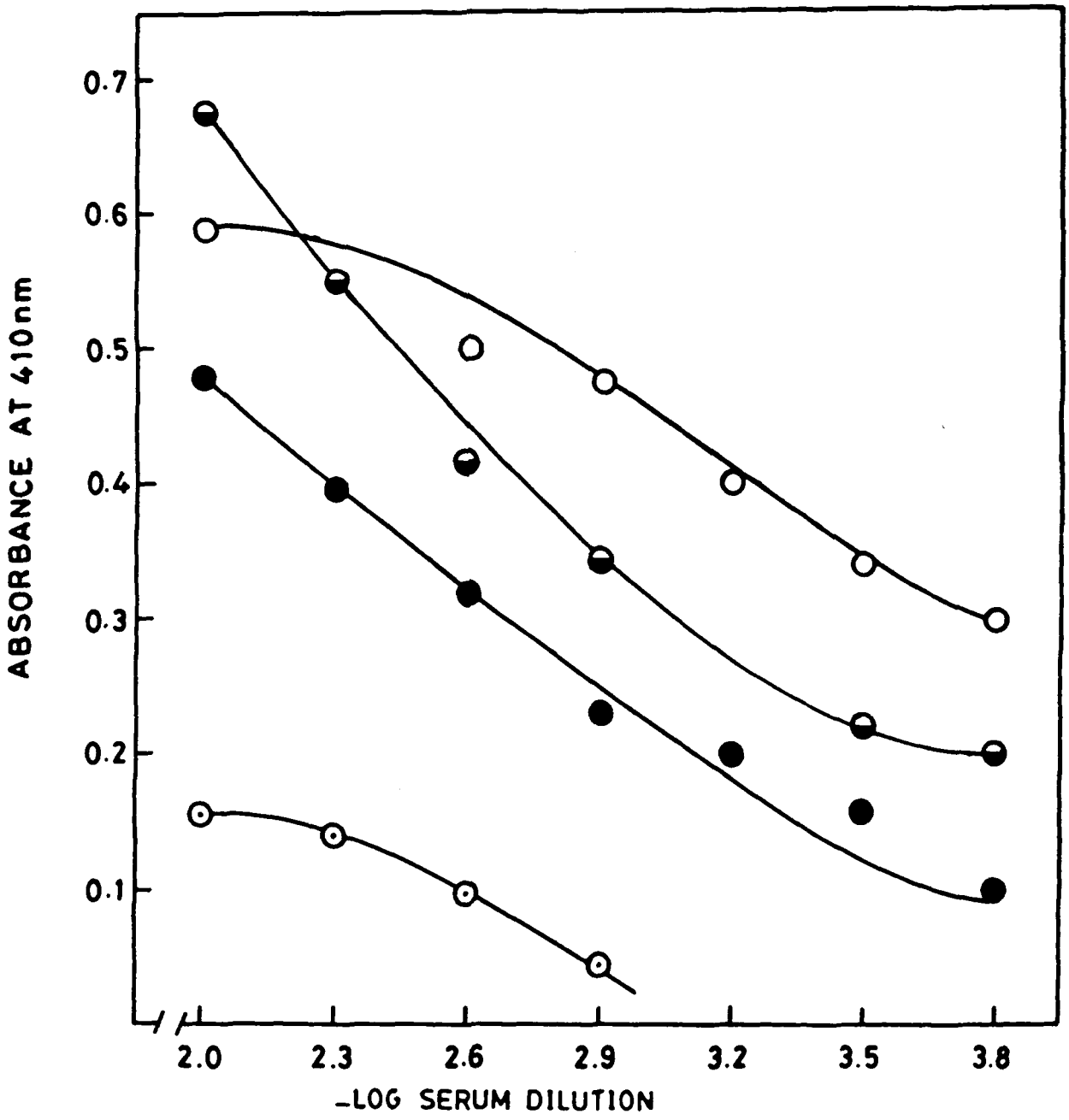

Figure 4. Direct binding ELISA of SLE serum.

(-○-) SLE serum 4 , (-◒-) SLE serum 5
(-●-) SLE serum 6 , (-⊙-) Normal human sera

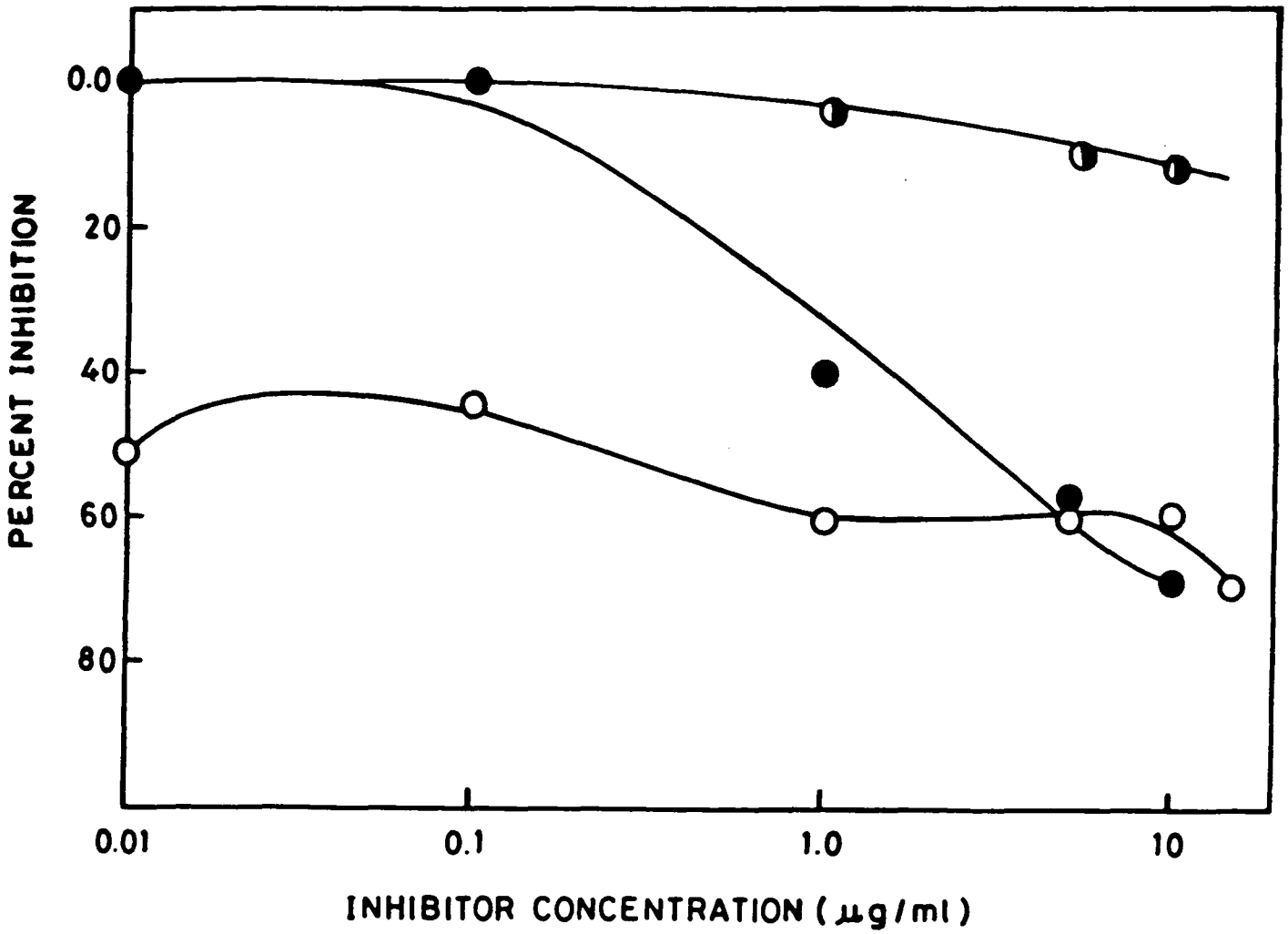

Figure 5. Inhibition ELISA of SLE anti-DNA antibodies. The microtiter plate was coated with native DNA (2.5 µg/ml). The competitors were Native DNA (-●-), ssDNA (-○-) and RNA (-◐-).

evident from the binding data (Fig. 5), although heat denatured DNA (ssDNA) when employed as an inhibitor exhibited the same magnitude of binding as was observed with the native DNA. However, the amount of inhibitor required to eliminate fifty percent antibody binding was reduced considerably. In this case, fifty percent inhibition was recorded at an inhibitor concentration of 0.2 µg/ml. Thus the result indicates that the SLE sera opted for our study had autoantibodies which were 10-fold more specific for ssDNA than for native DNA, as judged on the basis of 50 percent inhibition. In contrast to native DNA and ssDNA, RNA was found to be non-inihibiting (Fig. 5).

Intracellular proteins synthesized by the pathogenic strain $H_{37}Rv$. of Mycobacterium tuberculosis were employed as antigens to probe the possible role of environmental infectious agents in autoantibody production in SLE. Mycobacterium tuberculosis ($H_{37}Rv$) cultured on Sauton's medium was heat killed, harvested and subsequently the pelleted cells were suspended in sonicating buffer. Intracellular proteins from the bacilli (pellets) were liberated by sonication.

Prior to the binding studies against intracellular mycobacterial protein antigen(s), attempts were also made to probe the binding of SLE autoantibodies with total intact heat killed mycobacterial bacilli (unsonicated).

This was done in order to check the possible involvement of some proteins present on the outer surface of bacilli in anti-DNA antibody recognition. Plates coated with intact heat killed bacilli showed negligible binding with SLE anti-DNA autoantibodies (Fig. 6). As evident from the data , non-significant titer of 1:100 was observed. On the other hand, appreciably high binding was observed with the total mycobacterial sonicate (Fig. 7). No binding was observed with normal human sera. The specificity of SLE sera against total bacterial sonicate was checked by inhibition ELISA. A maximum of 80 percent inhibition in the antibody binding was observed at a maximum inhibitor concentration of 30 ug/ml. Fifty percent inhibition in the antibody activity was recorded at an inhibitor concentration of 4 ug/ml (Fig. 8).

Since apart from the intracellular proteins, the total mycobacterial sonicate (without centrifugation) employed in the above inhibition experiment possess mycobacterial DNA attached to the completely ruptured or sonicated bacilli as demonstrated by fluorimetric (Hoechst dye assay) as well as agarose gel electrophoresis (Fig. 9). Attempts were made to separate intracellular proteins from the sonicated bacilli. Microcentrifugation at high speed of the total sonicate yielded an extract referred to as Mycobacterium tuberculosis sonic extract (MTSE).

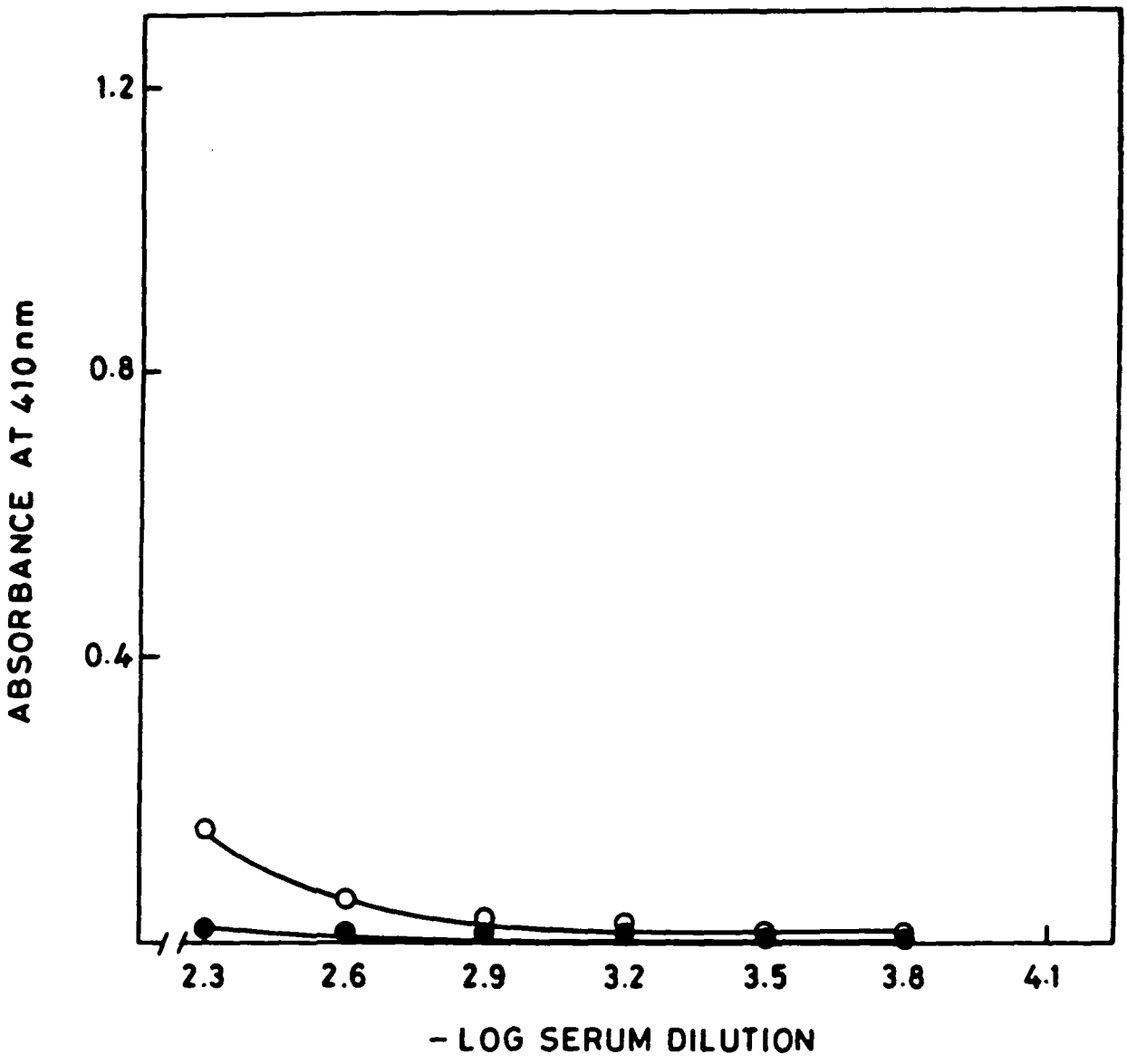

Figure 6. Direct binding ELISA of SLE anti-DNA antibodies. The microtiter plate was coated with heat killed bacilli (50 µg/ml).

(-○-) SLE serum
(-●-) Normal human sera

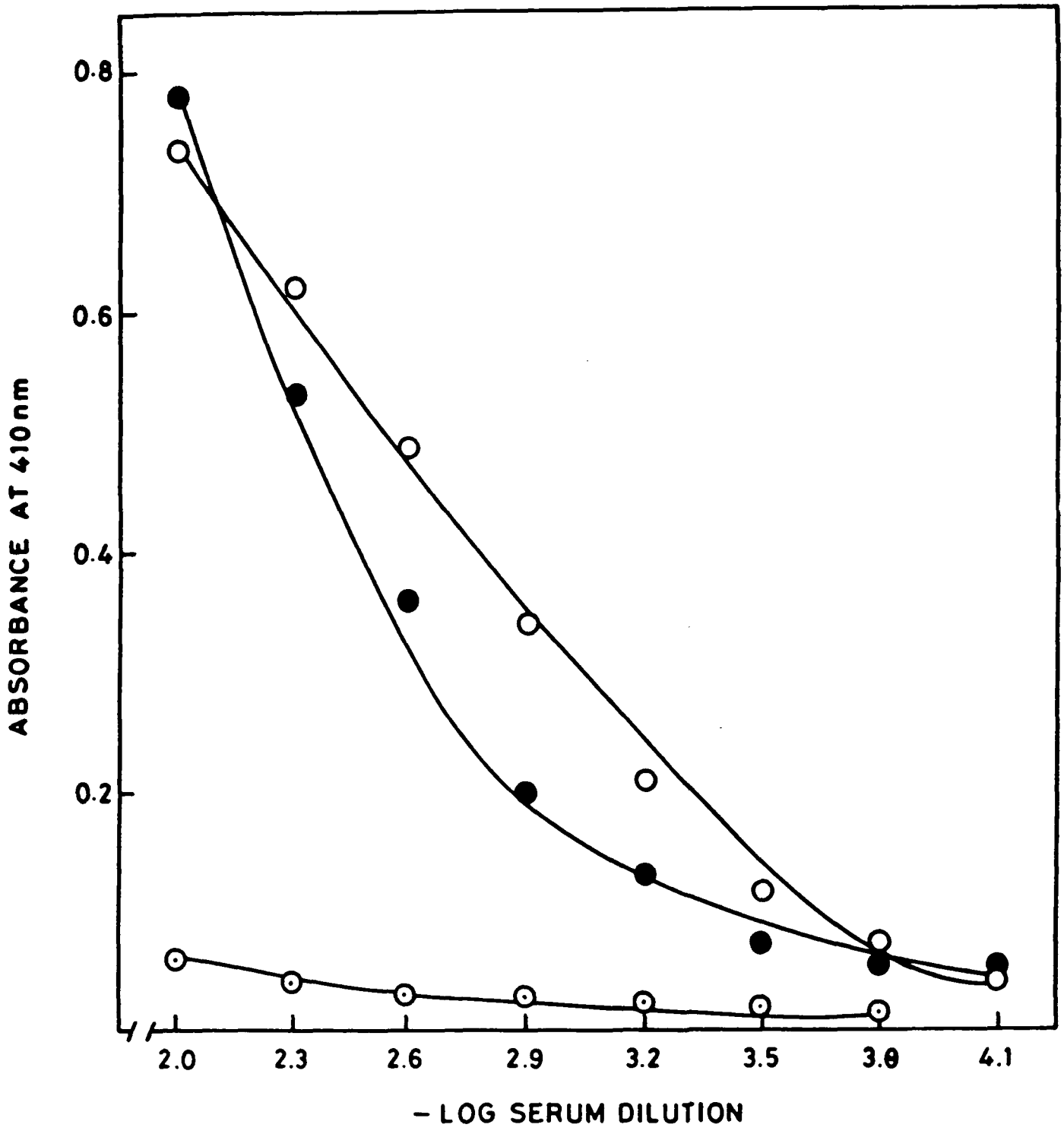

Figure 7. Direct binding ELISA of SLE anti-DNA antibodies. The microtiter plate was coated with total mycobacterial sonicate (50 µg/ml).

(–○–) SLE serum 1, (–●–) SLE serum 2
(–⊙–) Normal human sera

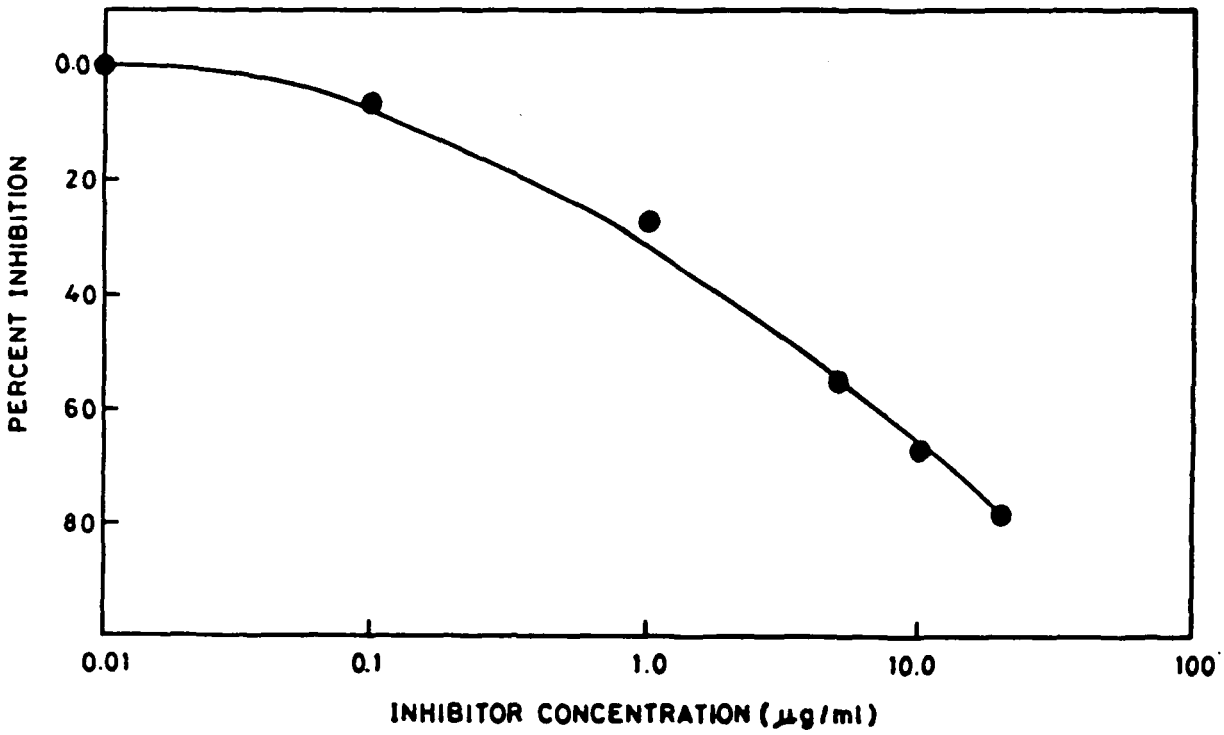

Figure 8. Inhibition ELISA of SLE antibodies binding with total mycobacterial sonicate. The microtiter plate was coated with total mycobacterial sonicate (50 µg/ml).

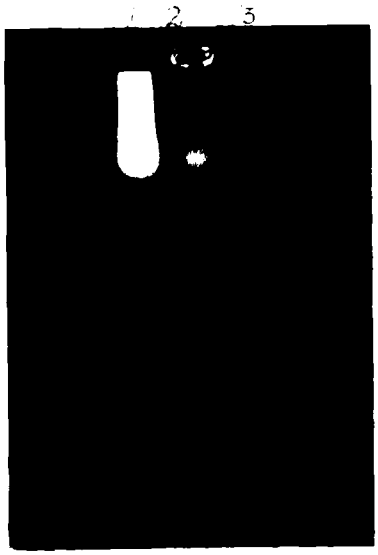

Figure 9. Agarose gel electrophoresis of intracellular and secreted mycobacterial proteins.

Lane 1 - native DNA
Lane 2 - sonicated bacilli
Lane 3 - secreted proteins

Colorimetric determinations of DNA by diphenylamine reagent were negative with MTSE, thereby indicating the absence of DNA in the mycobacterial sonic extract. Direct binding ELISA on microtiter plate coated with the DNA-free MTSE exhibited high binding with anti-DNA antibodies (Fig.10). When employed as inhibitor in competition ELISA, as illustrated in figure 11, a maximum of 82 percent inhibition in the antibody binding was observed at a maximum inhibitor concentration of 30 µg/ml. Fifty percent elimination in the antibody binding was recorded at an inhibitor concentration of 3.4 µg/ml.

The IgG was isolated from SLE serum by employing Protein A-Sepahrose column chromatography (Fig. 12). The homogeneous character of the isolated IgG was ascertained by polyacrylamide gel electrophoresis (Fig. 13). The electrophoretically pure IgG was used for further studies. Direct binding ELISA on plates coated with sonic extracts (MTSE) showed nearly the same magnitude of binding with SLE IgG as was observed with the serum (Fig. 14). With the sonic extract (MTSE), inhibition curves showed a maximum inhibition of 84 percent in the SLE autoantibody (IgG) binding (Fig. 15). As evident from the binding data (Fig. 15), fifty percent elimination in the antibody binding was recorded at an inhibitor concentration of 1.2 µg/ml. The results indicate the high specificity of SLE IgG for

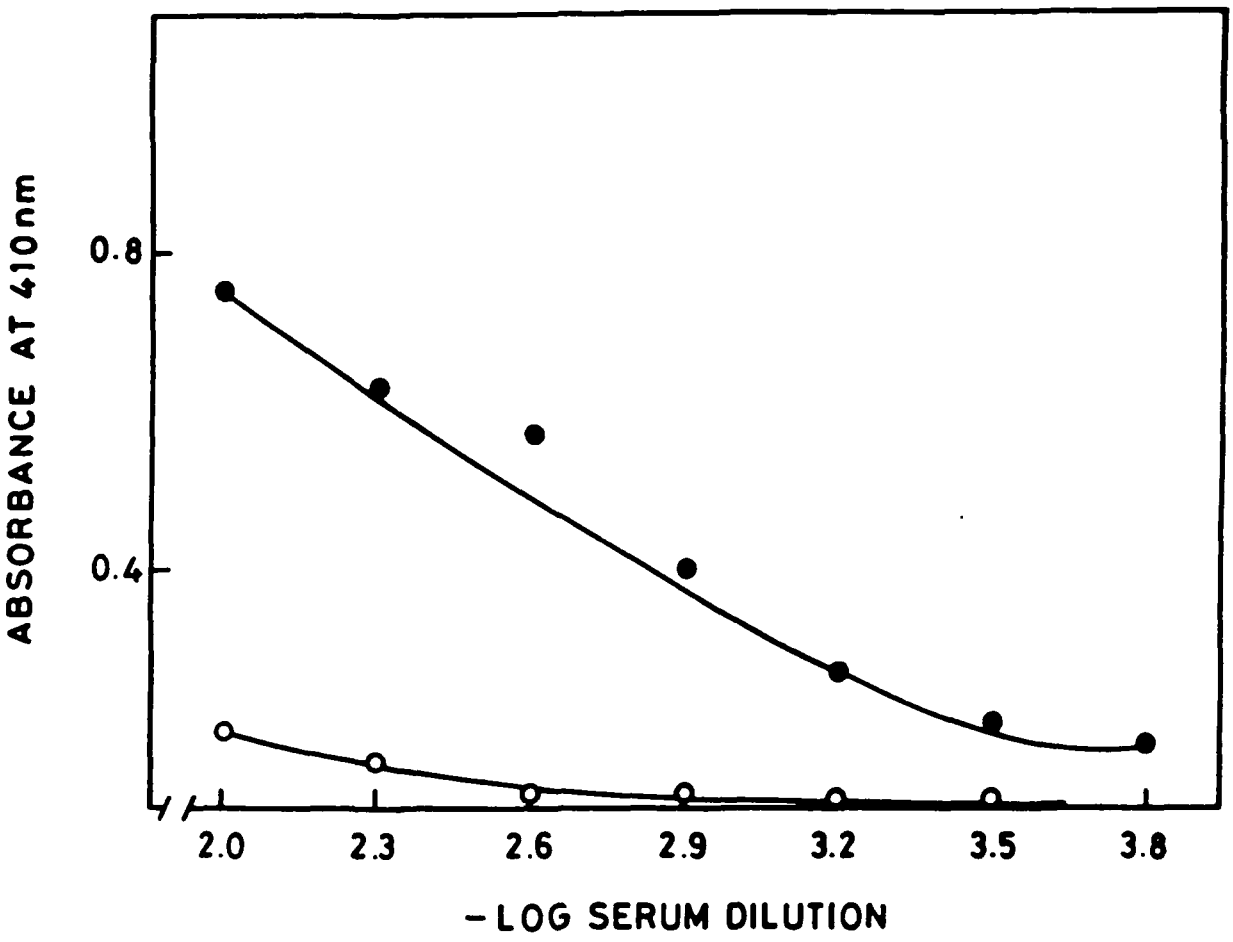

Figure 10. Direct binding ELISA of SLE anti- DNA antibodies. The microtiter plates were coated with MTSE (50 µg/ml).

(—●—) SLE serum
(—○—) Normal human sera

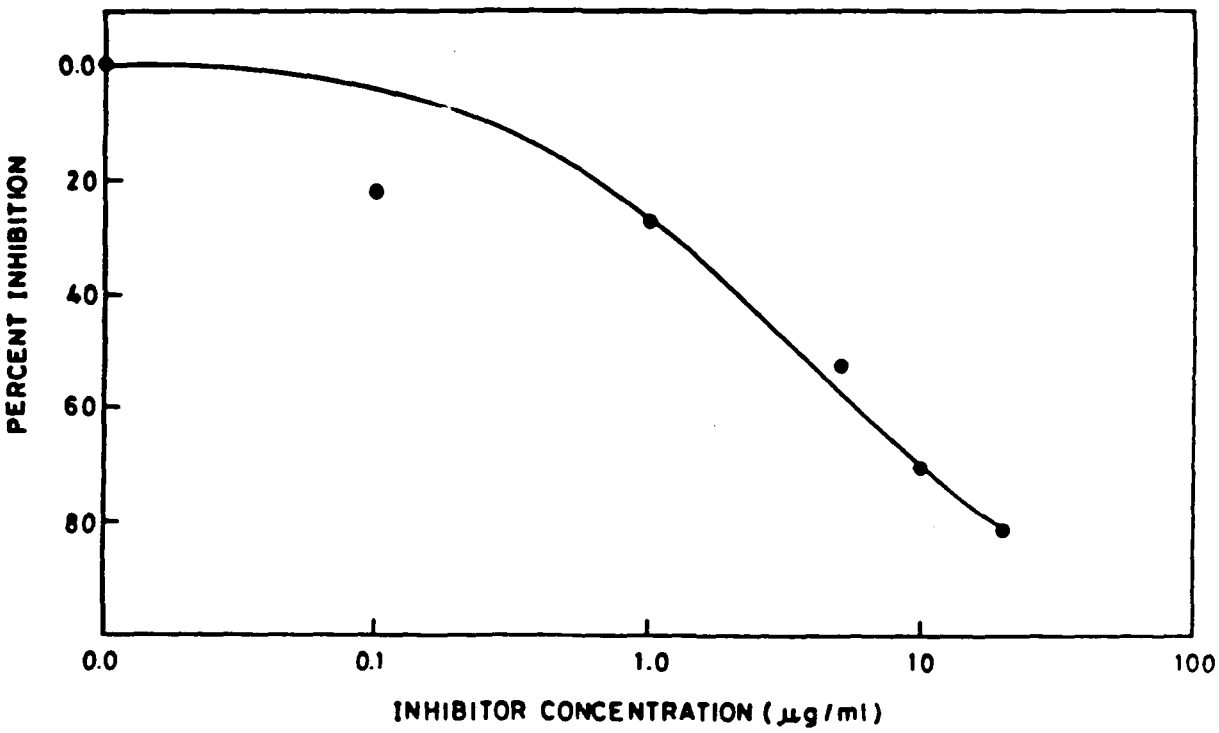

Figure 11. Inhibition ELISA of SLE anti-DNA antibody binding with MTSE. The microtiter plate was coated with MTSE (50 µg/ml).

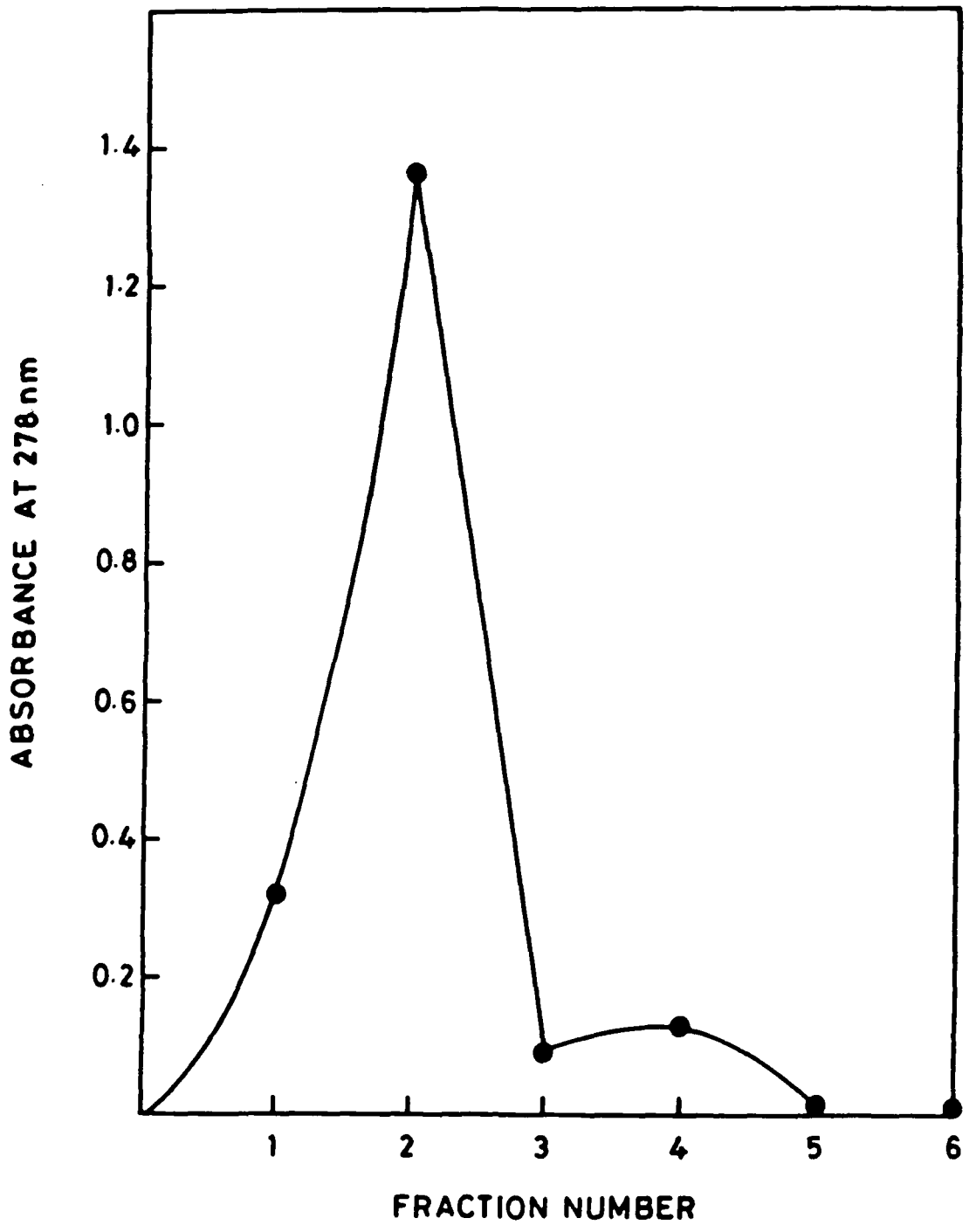

Figure 12. Elution profile of SLE IgG by Protein A-Sepharose column. High titer anti-DNA antibodies were applied onto the column and bound material was eluted with 0.58 percent acetic acid in 0.85 percent sodium chloride. Fractions of 3 ml each were collected in 1 ml of 1 M Tris-HCl, pH 8.5.

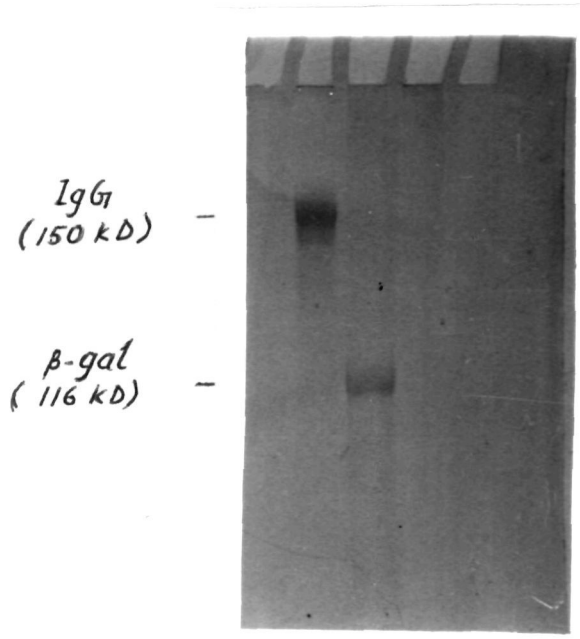

Figure 13. SDS-polyacrylamide gel electrophoresis of purified SLE IgG.

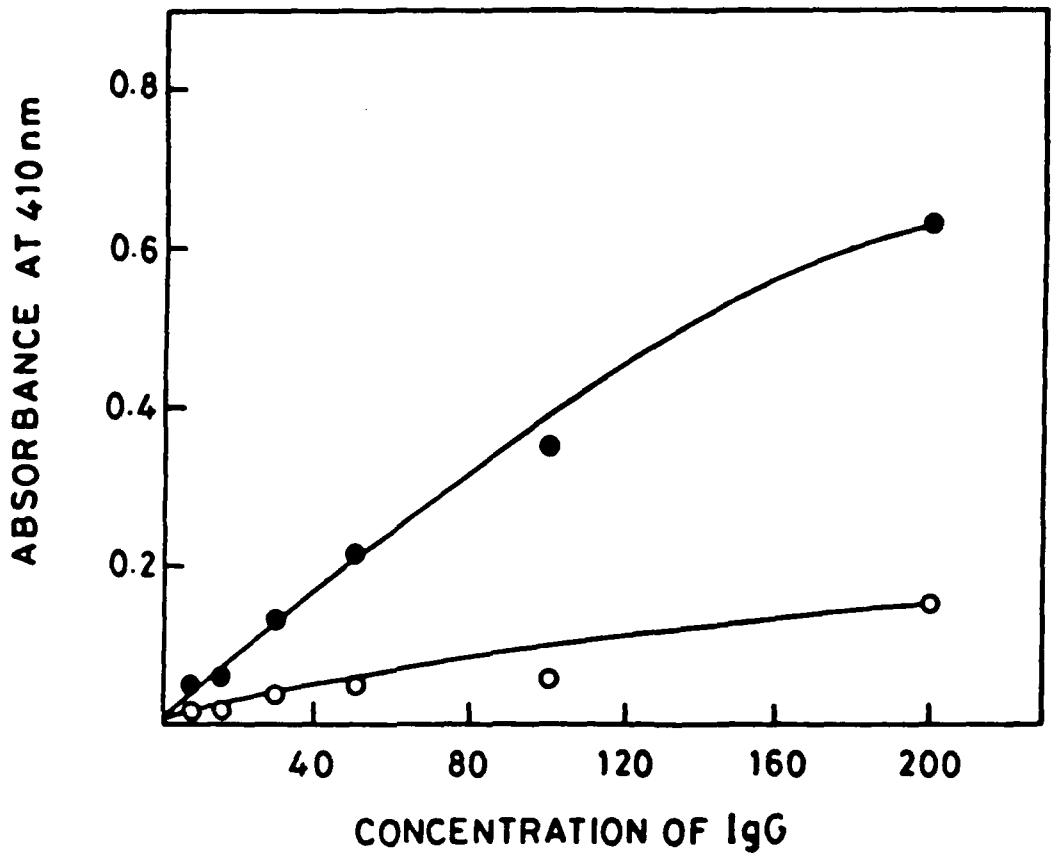

Figure 14. Direct binding ELISA of protein A-Sepharose purified SLE IgG with MTSE. The microtiter plate was coated with MTSE (50 µg/ml).

(—●—) SLE IgG
(—○—) Normal human IgG.

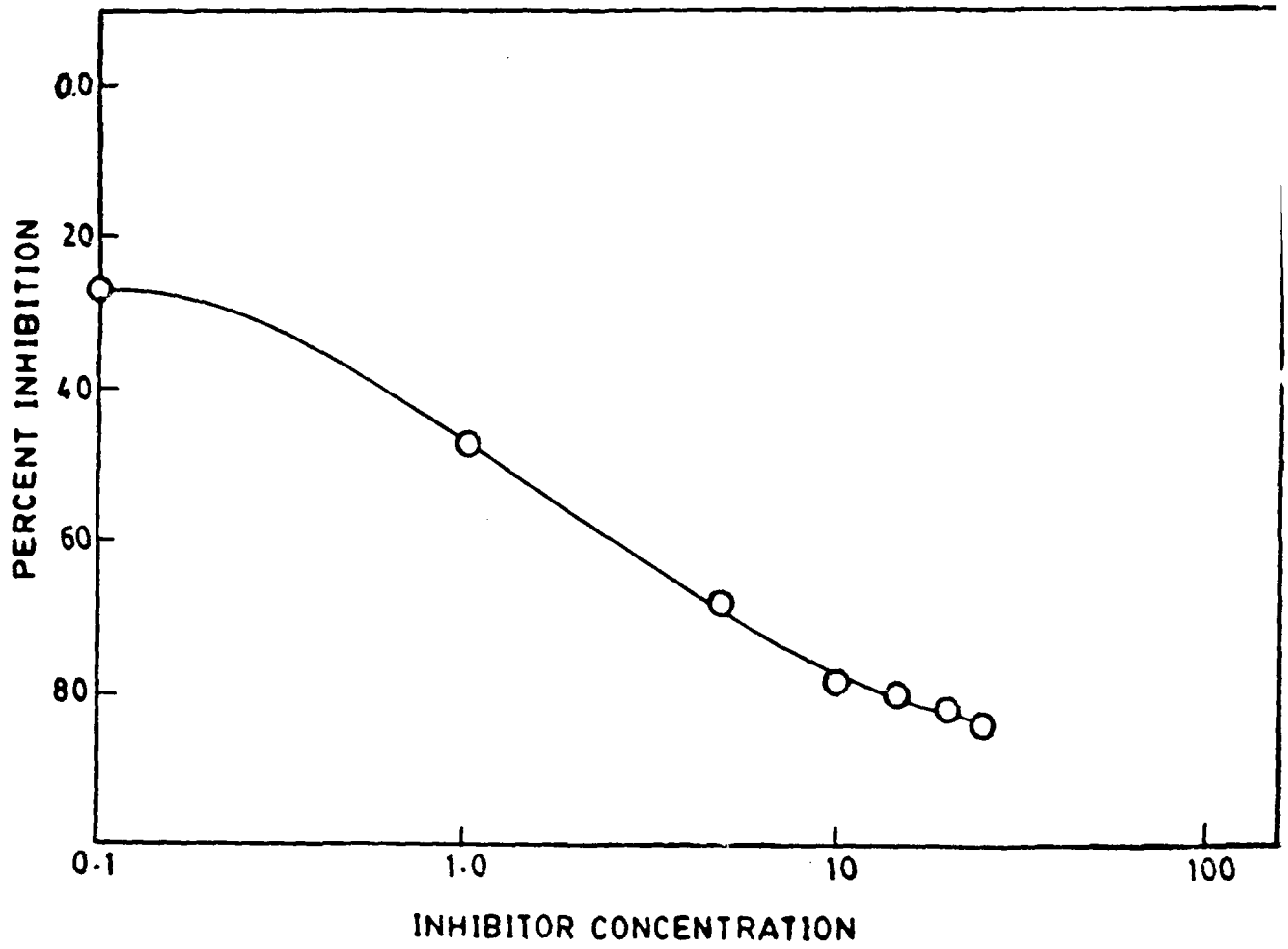

Figure 15. Inhibition ELISA of purified SLE IgG with MTSE. The microtiter plate was coated with MTSE (50 µg/ml).

the protein antigens in MTSE.

Mycobacterial 70 kD heat shock protein (hsp 70) was isolated from the sonicated bacilli of Mycobacterium tuberculosis ($H_{37}Rv$) by electroelution from an electrophoresed 5-20% gradient SDS-polyacrylamide gel. The results of gradients SDS-PAGE for sonicated bacilli as well as of the electroeluted 70 kD heat shock protein are depicted in Fig. 16a and 16b respectively. The electroeluted mycobacterial hsp 70 kD was employed in ELISA as well as Western blotting. Direct binding ELISA with plates coated with hsp 70 kD exhibited a binding of high magnitude by protein A-Sepharose isolated SLE IgG (Fig. 17). Once again, the binding of normal human IgG was of very low magnitude (Fig. 17). The inhibition studies (Fig. 18) revealed a maximum of 88 percent inhibition in the SLE IgG binding with hsp 70 kD at a maximum inhibitor concentration of 20 µg/ml. Only 0.8 µg/ml of inhibitor (hsp 70 kD) was required to eliminate fifty percent of SLE IgG activity (Fig. 18). The results thus indicate the high specificity of SLE IgG towards mycobacterial 70 kD heat shock protein. The binding characteristics of the SLE autoantibodies against various nucleic acids and mycobacterial antigens are given in Table 1.

In order to substantiate the above results, Western blot assay was also carried out so as to probe the

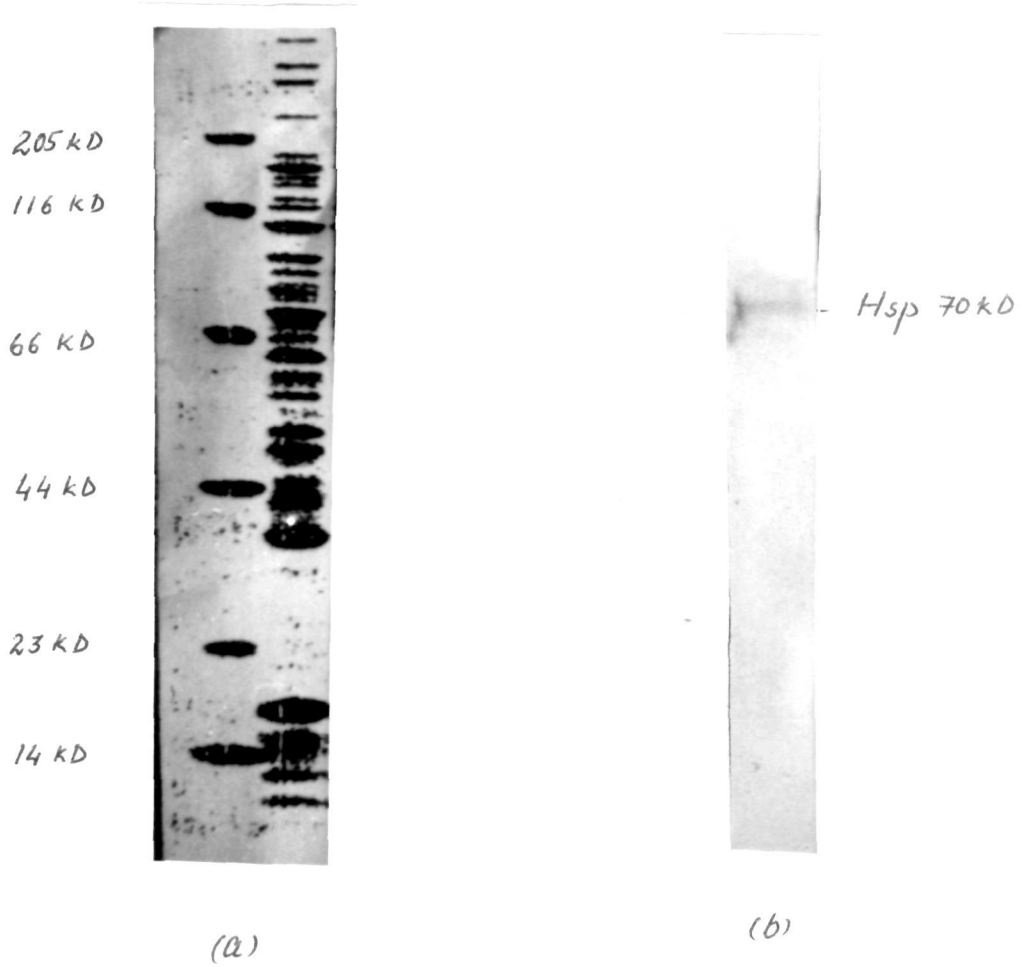

Figure 16 **(a)** SDS-PAGE of total mycobacterial sonicate on 5-20 percent gradient gel **(b)** SDS-PAGE of electroeluted 70 kD heat shock protein on 5-20 percent gradient gel.

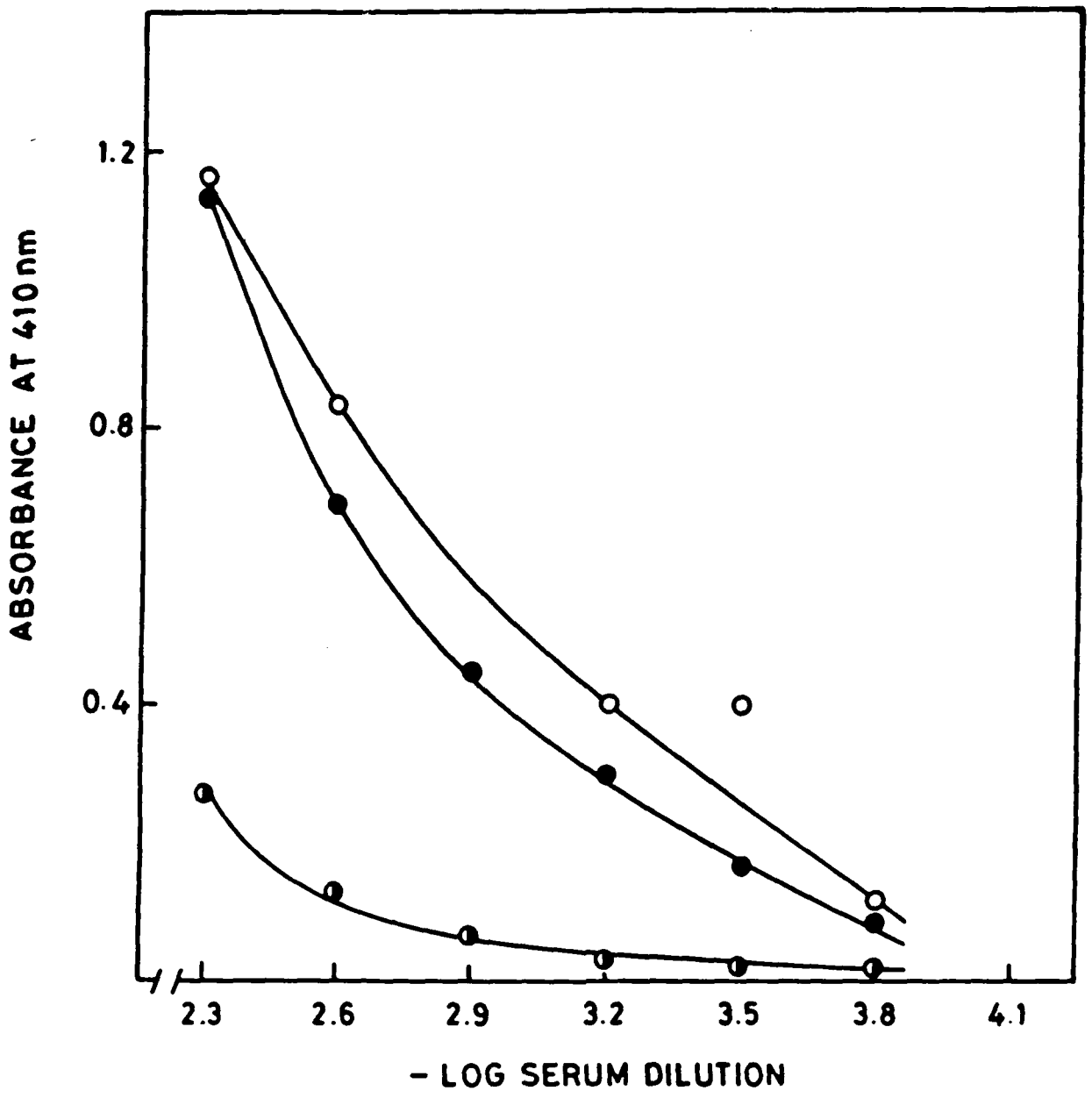

Figure 17. Direct binding ELISA of protein A-Sepharose purified SLE IgG on plate coated with hsp 70 kD.

(-○-) SLE serum, (-●-) SLE IgG and
(-◑) Normal human IgG

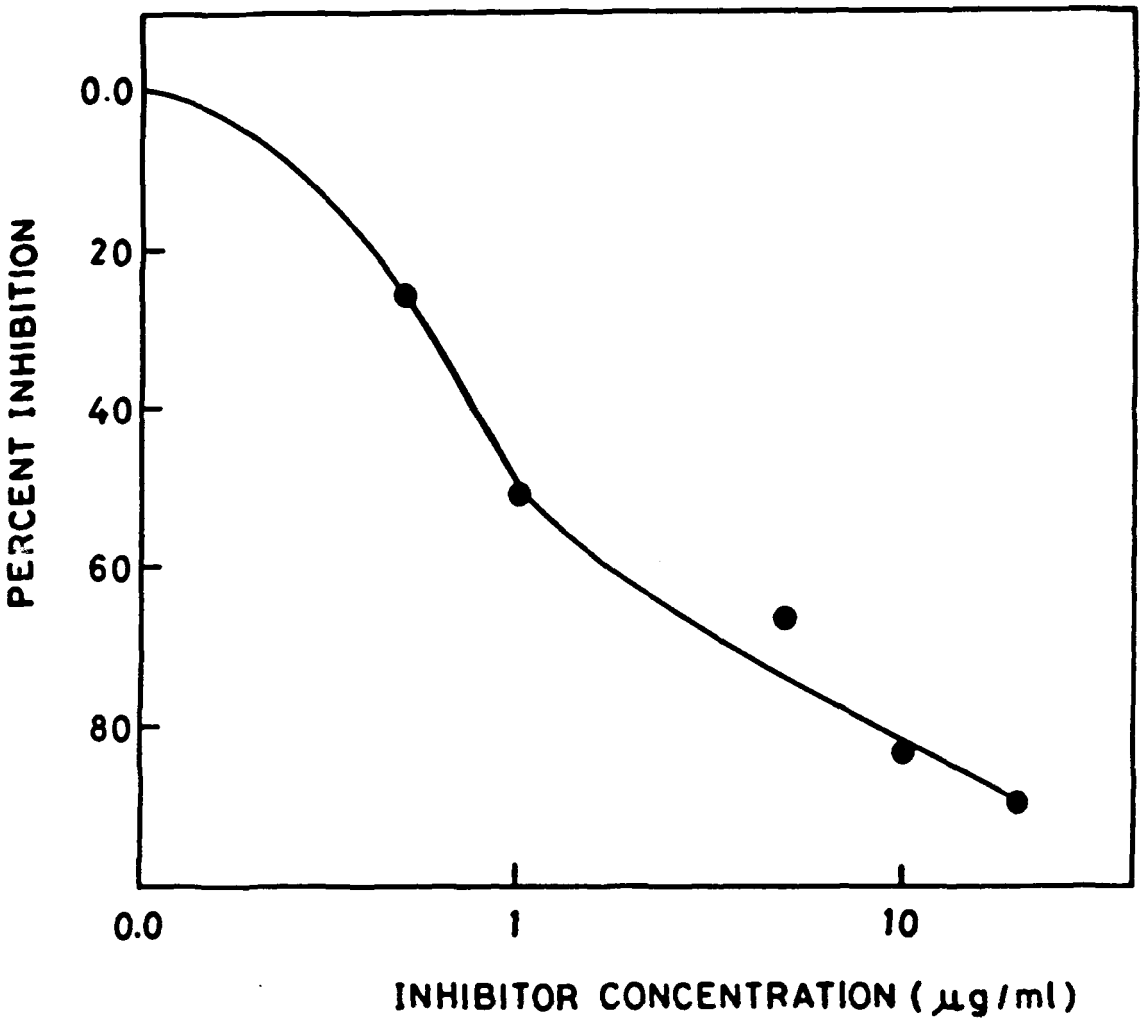

Figure 18. Competition immunoassay of SLE anti-DNA antibody by hsp 70 kD on plate coated with hsp 70 kD.

Table - 1

Competition Inhibition Data of various inhibitors with SLE serum and its isolated IgG.

Source of antibody	Inhibitor	Inhibitor concentration (µg/ml)	Concentration for 50% inhibition (µg/ml)	Maximum inhibitior (%)
SLE serum	dsDNA	0 - 10	2	69
	ssDNA	0 - 15	0.2	70
	RNA	0 - 10	-	15
	total sonicate	0 - 30	4.0	80
	MTSE	0 - 30	3.4	82
SLE IgG	MTSE	0 - 25	1.2	84
	hsp 70	0 - 20	0.8	88

immunointeraction of SLE IgG with mycobacterial 70 kD heat shock protein. Prior to Western blot analysis, SLE IgG was subjected to immunoaffinity purification on a column of DNA linked to polylysyl-Sepharose 4B matrix (Fig. 19). The above affinity purified SLE IgG on DNA-matrix was employed in Western blot analysis. The results depicted in Figure 20 indicated the strong antigen-antibody interaction undergone between 70 kD heat shock protein and immunoaffinity purified SLE IgG.

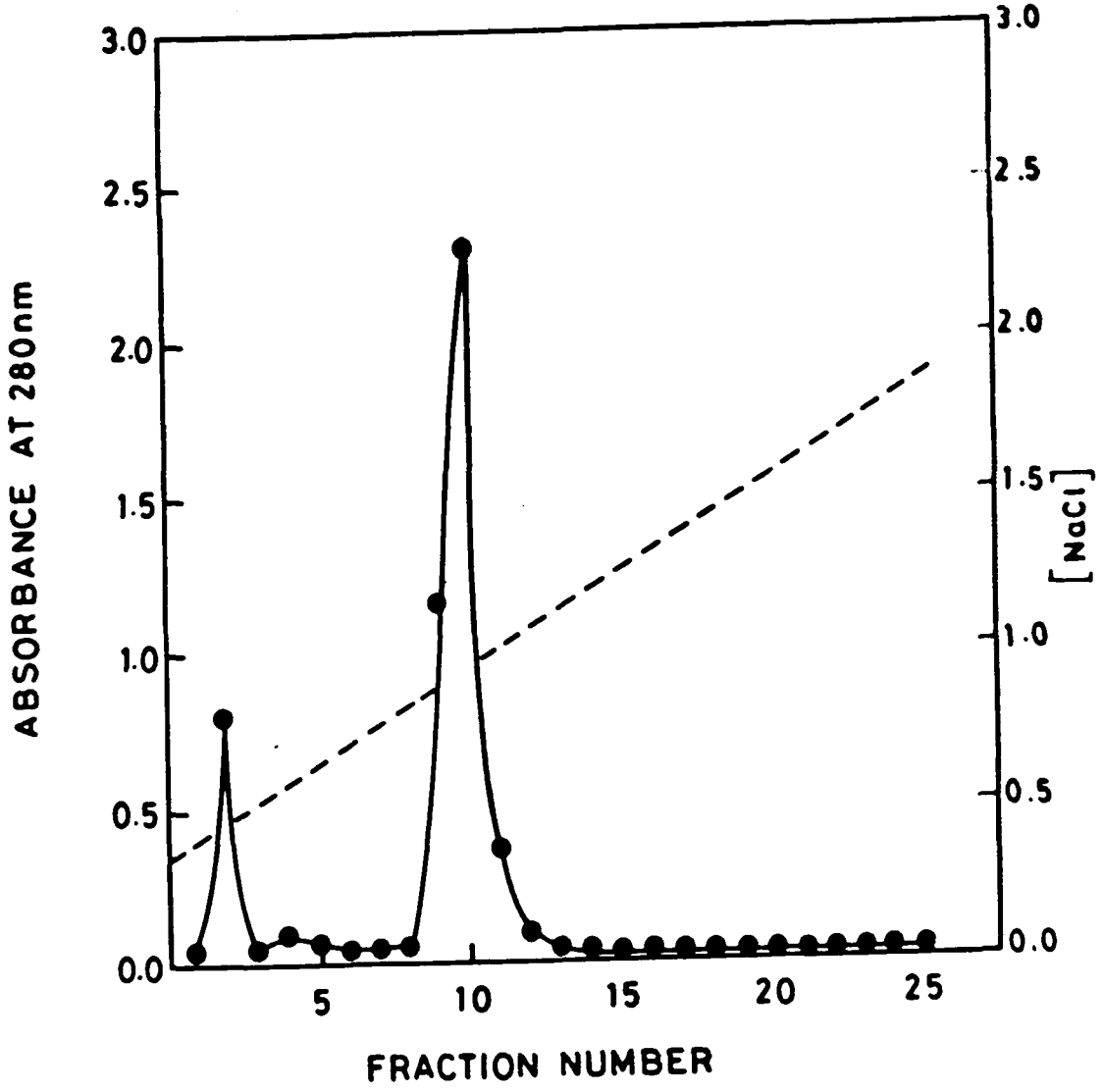

Figure 19. Immunoaffinity purification of anti-DNA antibodies on DNA- [polylysyl - Sepharose 4B] column. Anti-DNA positive SLE serum was passed through the column and bound material was eluted with linear ionic strength gradient (-----) of sodium chloride (0.15 to 3 M) in 0.01 M sodium phosphate buffer, pH 7.4.

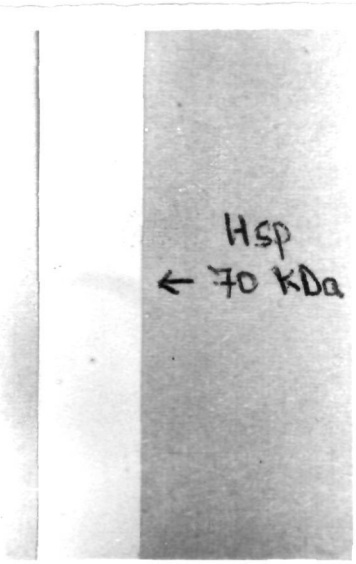

Figure 20. Western blotting of hsp 70 kD with affinity purified SLE IgG.

Discussion

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by the presence of heterogenous population of circulating antibodies of intracellular proteins and nucleic acids. These autoantibodies have specificities for nucleic acids (RNA and DNA), histones and ribonucleoproteins (Tan, 1989). There are also evidences that anti-DNA antibodies cross react with epitopes on infectious agents and that anti- DNA antibodies may be very similar in structure to anti-bacterial antibodies. (Hahn and Tsao, 1993). Questions about the mechanism leading to the formation of anti-DNA antibodies in SLE, their specificity as well as their relationship with antibodies directed towards other self constituents is still unsolved (Harley and Scofield, 1991; Brendel et al., 1991). In view of its poor immunogenicity native DNA is unlikely to be involved in SLE pathogenesis (Pisetsky et al., 1990). Thus, some other structure appears to be the trigger of autoantibody production whereas DNA might only be a crossreactive antigen. These structures include DNA modified with drugs, reactive oxygen species, nucleic acids of bacterial origin, DNA-psoralen crosslinks, B-estradiol linked DNA and Poly (lysine- glutamate) complexes (Caroll et al., 1985; Hasan et al., 1991; Ara et al., 1993; Alam et al., 1993;

Moinuddin and Ali, 1994; Arif et al., 1994). Native DNA is no longer regarded as the antigen initiating the disease mainly because immunization with native DNA does not induce SLE like symtoms.

The possible association of Mycobacterium tuberculosis and human autoimmune diseases have been indicated (Mclean et al., 1990) but their role in its pathogenesis is not yet clear. Recent studies indicate the possible involvement of mycobacterial heat shock proteins (hsps) acting as antigens for autoantibody production. (Winfield and Jarjour, 1991). Because infection entails " stress" for both the microorganism and the host (Buchmeier and Hefron, 1990) increased synthesis and altered expression of extremely similar sets of autologous and highly immunogenic foreign molecules occur during times of active immune responses. Mechanistically, therefore, infection would appear to be an ideal stimulus for the emergence of autoreactive T-cell and B-cell responses against host stress proteins.

In the present study attempts have been made to probe the binding of SLE anti-DNA autoantibodies with mycobacterial intracellular proteins as well as 70 kD heat shock protein synthesized by Mycobacterium tuberculosis ($H_{37}Rv$). The binding studies would help in elucidating the possible involvement of heat shock proteins in the

autoantibody production in SLE. Sera of SLE patients were assayed for anti-DNA antibodies by employing enzyme linked immunosorbent assay (ELISA). The immunocrossreactivity of SLE autoantibodies against mycobacterial heat shock proteins was analysed by ELISA and Western blotting. The selected SLE sera exhibiting high titer anti-DNA antibodies as revealed by direct binding ELISA were further subjected to specificity ascertation by employing competition inhibition ELISA. The inhibition ELISA results revealed for the presence of a greater magnitude of ssDNA specific autoantibodies in the opted SLE sera in comparison to native DNA. Thus, the binding data is suggestive for the presence of ten fold more specific autoantibodies directed against secondary structure than against the negatively charged backbone.

Anti-DNA antibodies have been found to cross react with membrane associated proteins (Jacob et al., 1984). Thus prior to binding studies, attempts were also made to probe the binding of SLE autoantibodies with total intact heat killed mycobacterial bacilli. This was done in order to check the possible involvement of some proteins present on the outer surface of mycobacterial bacilli in the interaction process. The binding results obtained with intact bacilli were found to be negative, thereby omitting the possibility of bacilli surface antigens in the

interaction process. After ascertaining the non-reactivity of SLE autoantibodies towards bacilli surface protein(s), attempts were made to probe the binding with intracellular mycobacterial proteins. Thus, the heat killed intact bacilli were ruptured by sonication in order to liberate the intracellular proteins. Interestingly, the binding data showed the SLE autoantibodies to possess a remarkably high specificity for intracellular mycobacterial proteins present in the total sonicate. The binding results are thus suggestive for the presence of autoantibodies in SLE sera to possess immunoreactivity towards intracellular proteins and not towards mycobacterial surface protein(s).

However, apart from intracellular protein(s), the total mycobacterial sonicate possess mycobacterial DNA attached to the sonicated bacilli. Therefore, the total sonicate was microfuged at high speed in order to separate the intracellular proteins from DNA. The mycobacterium tuberculosis sonic extract (MTSE) resulted as a consequence of microfugation of the total sonicate was found to be negative for DNA as demonstrated by fluorimetric estimation and agarose gel electrophoresis followed by staining with ethidium bromide. The MTSE so produced having exlusively intracellular proteins was employed in further binding studies. The competition-inhibition studies exhibiting a remarkably high binding

with MTSE is further supportive for the above argument that SLE autoantibodies possess appreciable immunoreactivity towards mycobacterial intracellular protein(s). The same magnitude of binding was observed with isolated SLE IgG thereby indicating the high specificity of SLE IgG for intracellular protein antigens in MTSE.

The 70 kD family of heat shock proteins is one of the most prominent classes of heat shock proteins. A large portion of the immune response to infecting mycobacteria appear to be directed at two particular proteins mainly 70 kD and 65 kD. (Shinnick et al., 1988). Therefore, in the light of the above view, hsp 70 kD was isolated from the sonicated bacilli of Mycobacterium tuberculosis($H_{37}Rv$). The 70 kD protein showed high magnitude of binding with Protein-A Sepharose isolated IgG. High specificity of SLE IgG towards hsp 70 is evident from the inhibition ELISA results. Further evidence for the anti-DNA-hsp 70 kD interaction was obtained by Western blotting by employing immunoaffinity purified anti-DNA IgG. The strong recognition of affinity purified anti-DNA IgG with hsp 70 kD further strengthens the above observations. These observations are important in view of the multifactorial pathogenesis of human SLE (Wick et al., 1987). The retention of crossreactivity of autoantibodies with hsp 70

kD and mycobacterial sonic extract after immunoaffinity purification further augments the idea of an alternate antigen for the induction of autoantibodies cross reactive with native DNA. Since mankind is quite susceptible to mycobacterial infection, the possible involvement of mycobacterial antigen(s) (hsp or non hsp) in SLE autoantibody induction can be inferred from our observations. Thus, the role of intracellular mycobacterial protein antigen(s) in the pathogenesis of SLE needs further investigation.

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